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TESI DI LAUREA

Investigating the role of m6A RNA methylation in stem cell regulation and regeneration in the model flatworm *Macrostomum lignano*

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Summary of the thesis project (Italian)

La metilazione m6A è una delle modifiche più diffuse e conservate che troviamo principalmente a livello dell'mRNA.
Si tratta della deposizione di un gruppo metilico a livello del sesto azoto dell'adenosina, che viene deposto durante la formazione del trascritto, principalmente nella regione prossima al 3' e vicino al codone di stop, ma distribuito lungo tutta la lunghezza della molecola.
La regolazione di questa modifica avviene grazie alla presenza di tre classi di proteine: i "writers" si occupano di depositare il gruppo metile, i "readers" riconoscendo il pattern di metilazione nella molecola sono responsabili di stabilirne il destino, mentre gli "erasers" lavorano in equilibrio dinamico con i writers, occupandosi della rimozione del gruppo metile.
Questa modifica è strettamente coinvolta alla regolazione di proliferazione e differenziazione delle cellule staminali, ed è infatti fattore chiave in importanti processi come ematopoiesi e sviluppo embrionale nei mammiferi, oogenesi e determinazione sessuale in *Drosophila melanogaster* e meiosi in *Saccharomyces cerevisiae*, ma gioca un ruolo fondamentale anche nei processi di rigenerazione ed invecchiamento.

L'obiettivo di questo progetto di tesi era di caratterizzare la regolazione della metilazione m6A, (partendo da alcuni dati precedentemente ottenuti ma non ancora pubblicati) con focus sulle proteine "writers", nelle cellule staminali di un organismo modello emergente, che presenta importanti capacità rigenerative e di longevità: il platelminta *Macrostomum lignano.* Nel dettaglio, i geni considerati sono stati *Mlig-mettl3, Mlig-mettl14, Mlig-mettl16* (metiltransferasi), *Mlig-wtap, Mlig-rbm, Mlig-kiaa, Mlig-hakai* (proteine accessorie).

M. lignano è un verme piatto di 1-1,5 mm di lunghezza che risulta ottimale come organismo modello, grazie ad alcune caratteristiche specifiche: oltre a presentare un corpo trasparente che si presta alla creazione di linee transgeniche con tag fluorescenti visualizzabili in vivo, se amputato, è in grado di rigenerare qualsiasi tipo di tessuto ed organo, partendo dalla regione della testa, grazie alla presenza di cellule staminali adulte chiamate neoblasti. Si tratta di un ermafrodita in quanto presenta le gonadi di entrambi i sessi, ma non è in grado di autofecondarsi, e depone 1-2 uova al giorno allo stadio di zigote, che si prestano in maniera ottimale a manipolazioni geniche grazie a tecniche di microiniezione. *M.lignano*, infatti, è l'unico platelminta in cui è possibile applicare metodi di transgenesi. È stato osservato inoltre, come sia in grado di sopravvivere fino ad almeno due anni di vita, risultato sorprendente per un organismo delle sue dimensioni.

L'approccio di studio si è avvalso di metodi di silenziamento genico come RNA interference (RNAi), applicata attraverso il metodo di "soaking" adattato per *M. lignano*, in vermi adulti ed in fase di sviluppo, e di microiniezioni sulle uova; per mappare l'espressione dei geni in studio è stato invece utilizzato un protocollo innovativo di In Situ Hybridization (ISH).

La ISH ci ha permesso di visualizzare il pattern di espressione dei geni precedentemente identificato da analisi di sequenziamento, confermando la loro presenza nelle regioni caratterizzate da cellule in proliferazione, principalmente le gonadi e meno rappresentate anche le bande laterali popolate dai neoblasti. Nei vermi in rigenerazione, in particolare, si è potuto osservare la loro espressione nel cosiddetto "blastema" ovvero una zona presente nei pressi della coda, che si crea dopo l'amputazione ed è caratterizzata dalla presenza di neoblasti con un alto rate di proliferazione. Questa tecnica è inoltre stata fondamentale nel validare gli esperimenti di RNAi, in quando abbiamo potuto visualizzare una significativa deplezione del segnale conseguente al trattamento del silenziamento genico.

Per gli esperimenti di RNAi è stata utilizzata una linea transgenica che si prestava in maniera ideale allo studio delle dinamiche dei neoblasti, creata dal knock-in della proteina fluorescente mNeonGreen nel gene dell'istone H2AX, marker per cellule in proliferazione. I vermi di questa linea presentano, dunque, delle regioni di forte fluorescenza dove sono situate le cellule staminali, quindi in due bande presenti lateralmente rispetto alla lunghezza del corpo e nelle gonadi. Tuttavia, la proteina è presente all'interno del nucleo e quando le cellule differenziano questa non viene subito degradata, il segnale di conseguenza permane, anche se con minor intensità, anche nelle zone popolate da cellule differenziate, ovvero nell'area più interna del verme. Ciò risulta in un segnale particolarmente intenso ai lati del verme e nelle gonadi, che diviene sempre più debole verso le regioni interne e ci permette di visualizzare non solo la presenza dei neoblasti e delle cellule germinali ma anche delle cellule più differenziate.

Gli esperimenti di RNAi sono stati organizzati in tre studi, suddividendo vermi allo stadio embrionale, vermi adulti ed in rigenerazione e vermi in fase di sviluppo dalla schiusa delle uova fino all'età adulta.

Il knockdown a livello embrionale è stato eseguito iniettando negli zigoti la soluzione di dsRNA contro il gene di interesse, considerando un centinaio di uova per gene. Come controllo negativo, ho utilizzato un dsRNA contro *heh1,* un gene presente in *C. elegans* ed assente in *M.lignano,* mentre come controllo positivo il gene *Mlig-ddx39* codificante un fattore di trascrizione, il cui silenziamento provoca la morte anche in vermi adulti. Gli stessi controlli sono stati considerati per tutti gli esperimenti di knockdown.

Questo esperimento partiva dall'ipotesi che, se i geni per i writers sono espressi in questo stadio ed effettivamente concorrono alla proliferazione e differenziazione cellulare, lo sviluppo embrionale avrebbe dovuto interrompersi. I risultati hanno mostrato innanzitutto che una percentuale di uova (≈12%) non sono sopravvissute neanche nel controllo negativo, indice che l'iniezione di per sé è un evento traumatico per le cellule, mentre nel controllo positivo il 95% delle uova non è sopravvissuta. Nel resto dei geni la percentuale di embrioni che non sono stati in grado di terminare lo sviluppo è compresa tra il 10% e il 25%, ma dal test statistico (Kruskal-Wallis per distribuzioni non parametriche e Dunn's test per il confronto tra geni e il controllo negativo) non ci sono stati risultati significativi. L'interpretazione di questo esperimento tiene conto di alcune difficoltà tecniche, legate all'assenza di conferma relativa alla percentuale di uova iniettate con successo. L'unico riferimento a ciò lo troviamo nei risultati del controllo positivo in cui sembrerebbe che una percentuale molto alta di uova sia stata correttamente iniettata per cui possiamo ipotizzare che ciò sia avvenuto anche negli altri casi. Inoltre, nonostante i dati non siano risultati statisticamente significativi, non per forza l'RNAi non ha funzionato, ma questi risultati potrebbero essere il riflesso di una condizione biologica che è ancora a noi sconosciuta.

L'RNA interference nei vermi adulti ed in rigenerazione è avvenuta per aggiunta della soluzione di dsRNA nel medium in cui sono presenti i vermi. Dopo due settimane di trattamento è stata osservata perdita di segnale fluorescente partendo dalle zone interne e progredendo lateralmente nei vermi in omeostasi, in tutti i geni tranne *Mlig-mettl3, Mlig-mettl14* e *Mlig-hakai.* Contemporaneamente, sono stati osservati gli effetti del silenziamento durante la rigenerazione, in vermi amputati sotto la faringe, dopo una settimana di trattamento. Quest'ultimi sono risultati avere una rigenerazione più lenta che nel caso di *Mlig-rbm* e *Mlig-kiaa* non è stata completata, causando la morte dei vermi. Anche in questo caso nessun fenotipo particolare è stato osservato nei vermi trattai per *Mlig-mettl3, Mlig-mettl14* e *Mlig-hakai.* Sembrerebbe dunque che il knockdown inizialmente abbia interferito con la capacità dei neoblasti di differenziare e in ultimo nella loro capacità di proliferare. Tutti i vermi, una volta fermato il trattamento, sono stati in grado di ritornare ad uno stato fisiologico normale, tranne quelli sopracitati e gli adulti non amputati trattati per *Mlig-rbm,* che sono morti dopo una settimana dal termine del trattamento.

L'esperimento di silenziamento nei vermi in fase di sviluppo ha incontrato qualche problematica. I vermi, infatti, non sono inizialmente stati nutriti a sufficienza causando uno stato di denutrizione che, anche se per loro sopportabile, ha probabilmente causato una variazione nell'espressione genica, interferendo con

l'esperimento. *Macrostomum lignano* è infatti in grado di sopravvivere anche per lungo tempo in condizioni di denutrizione, riducendo le proprie dimensioni e atrofizzando o regredendo del tutto le gonadi. Ciò è possibile grazie ad eventi di apoptosi e ad una riduzione del frequenza di proliferazione cellulare. Considerando che i geni coinvolti nella metilazione m6A sono importanti per la regolazione della proliferazione, è logico ipotizzare che la condizione di denutrizione abbia interferito con la loro espressione. I risultati hanno mostrato la manifestazione di tre fenotipi: vermi detti +signal/+gonads che hanno raggiungo lo stadio di età adulta con una dimensione normale, gonadi completamente sviluppate ed un pattern di fluorescenza regolare; vermi detti +signal/-gonads, caratterizzati da un segnale di fluorescenza normale ma con dimensioni ridotte e mancanza di gonadi; un ultimo fenotipo detto -signal/-gonads in cui oltre ad avere dimensioni ridotte, presentano
un'importante riduzione del segnale e assenza di gonadi. Quest'ultimo fenotipo è il più interessante, in quando non è mai stato osservato precedentemente come associato ad una condizione di denutrizione (al contrario del fenotipo +signal/-gonads) ed è molto probabilmente quello causato dall'effetto del knockdown, come la sua assenza nel controllo negativo e in *Mlig-mettl3* suggerirebbe ulteriormente. Anche in questo caso, la mancanza di segnale sembrerebbe dunque essere legata ad una incapacità dei neoblasti di rinnovarsi e differenziare.

I risultati ottenuti sembrano pertanto indicare che la metilazione m6A sia fondamentale nei processi di proliferazione e rigenerazione anche nel modello platelminta *Macrostomum lignano.* Tuttavia, non possedendo conferma che i dati degli esperimenti siano relativi ad effettivi cambiamenti della metilazione, posso solo dichiarare che il silenziamento delle proteine coinvolte nella deposizione di questa modifica epitranscrittomica interferisca significativamente nella regolazione dei neoblasti.

In conclusione, credo che gli esperimenti condotti abbiano contribuito ad una comprensione più profonda della regolazione m6A, sollevando interrogativi utili a perseguire la ricerca su questo campo.

Abstract

m6A methylation is one of the most common and conserved RNA modification primarily found in mRNAs. It is involved in important processes regarding cellular proliferation and differentiation such as development, regeneration and stem cell regulation. Recent studies have discovered that in the planarian flatworm model *Schmidtea mediterranea,* which is able of whole-body regeneration, the knock-down of some m6A machinery genes prevents regeneration after amputation, suggesting how this epitranscriptomic modification is fundamental for regeneration. Here, I present a study where we investigated the role of m6A methylation machinery proteins in regulating the activity of somatic stem cells called neoblasts, in another free-living flatworm model *Macrostomum lignano.* Starting from previous data, we explored this mechanism through experiments of RNA interference (RNAi) and In Situ Hybridization (ISH), on genes expressing major conserved components of m6A machinery during regeneration at different life stages of the worms. Results showed impaired neoblasts homoeostasis activity in adults and hindered regeneration in amputated worms, showing the importance of stem cells epitranscriptomics in the regulation of neoblasts. Moreover, A far less penetrant phenotype was observed in the treatment with hatchlings and not significant results were found by treating eggs.

Overall, these experiments offer new insights into the role of m6A machinery in *M. lignano's* neoblast activity, which understanding would significantly impact research on ageing and regeneration.

Chapter 1: Introduction

1.1 m6A as a spread and conserved modification

One of the most spread mRNA modifications in eukaryotic cells is the methylation of the sixth nitrogen of adenosine or N6-methyladenosine (m6A). It was first discovered in bacteria in 1955 mostly in ribosomal RNA, but later in the 1970s, thanks to the development of efficient RNA isolation methods, it was identified also in mammalian cells and its presence in mRNA was firstly observed(Sendinc and Shi 2023).

Since then, m6A has been more explored even though detailed studies of its functions did not begin until around 2012, when transcriptome-wide profiling of m^6A was made possible through antibody-based immunoprecipitation, followed by high-throughput sequencing (Yang et al. 2018). Now we know that it is present also in many eukaryotic species, including yeast, flatworms, plants, flies and mammals where it has been found in nearly all types of RNAs like mRNA, rRNA, snRNA and different species of regulatory RNAs. It plays a crucial role in many molecular processes including pre-mRNA splicing and nuclear export, mRNA degradation and stability, development and differentiation. In mRNAs, the modification is typically found clustered around the stop codon and within the 3' untranslated regions, localized in two slightly different consensus motifs "RRACH" and "DRACH" (D = U, A, or G; R = A or G; H = U, A, or C). However, these are not rare sequences in mRNAs, and most DRACHs/RRACHs are not methylated (Meyer et al. 2012; Sun et al. 2022) Instead, it is now believed that RNA secondary structure plays a key role in determining m^6A RNA modifications(Martinez De La Cruz, Darsinou, and Riccio 2023).

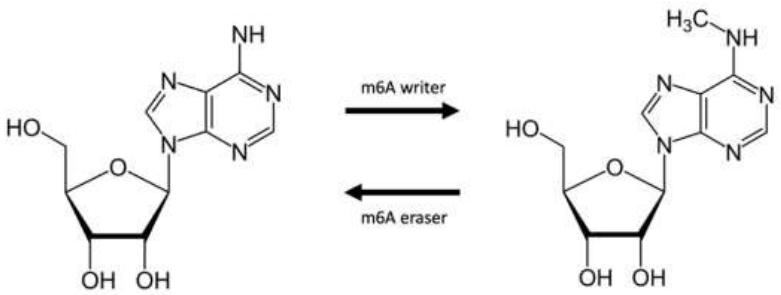


Fig. 1.1.1 m6A
Adenine with and without the methyl group on the 6th nitrogen.

1.1.1 m6A machinery

m6A regulation is dependent on three different “classes” of protein complexes: writers, readers and erasers.

m6A writers

The “writers” are all the proteins involved in the deposition of the methyl group, including the METTL3/METTL14/WTAP complex, RBM15, VIRMA/KIAA1429, HAKAI, and METTL16(Sun et al. 2022).

The methyltransferase-like 3 (METTL3) represents the sole catalytic core of a heterodimer with METTL14. It is the main responsible for the deposition of the methyl group thanks to the presence of an S-adenosyl methionine (SAM) which works as a donor substrate(Śledź and Jinek 2016). Severe phenotypes are observed after the knock-out of METTL3 which underscore the importance of this molecule in cellular homeostasis during development, cancer growth and viral infections. Moreover, METTL3 has recently been found also in the cytoplasm, where it can act as an m6A reader to regulate mRNA translation, however the mechanism that permits the switch from writer to reader and how it works as the latter is still unclear(Wei et al. 2022; Gupta and Qi 2023).

The Methyltransferase-like 14 (METTL14) is a secondary methyltransferase that doesn’t have a catalytic activity and possesses a degenerative active site. This could mean that METTL14 has lost its catalytic activity as it is suggested by some phylogenetic studies(Iyer, Zhang, and Aravind 2016). Its main role seems to be related to the complex/mRNA interaction and stabilization, allowing the METTL3 catalytic site to interact with the correct nucleotides(Ping et al. 2014; E et al. 2018).

Wilms’ tumour 1 associating protein (WTAP) is an accessory protein that connects with the METTL3/METTL14 heterodimer, and it is required for their localization in nuclear speckles. It cannot catalyse m6a modification since it lacks a conserved catalytic methylation domain, but it is still a really important member of this complex, indeed its deletion significantly affects m6A levels and physiological processes like embryonic differentiation.(Ping et al. 2014) METTL3/METTL14/WTAP (MMW complex) is therefore the core complex responsible for the m6a modification, which acts primarily on mRNA.

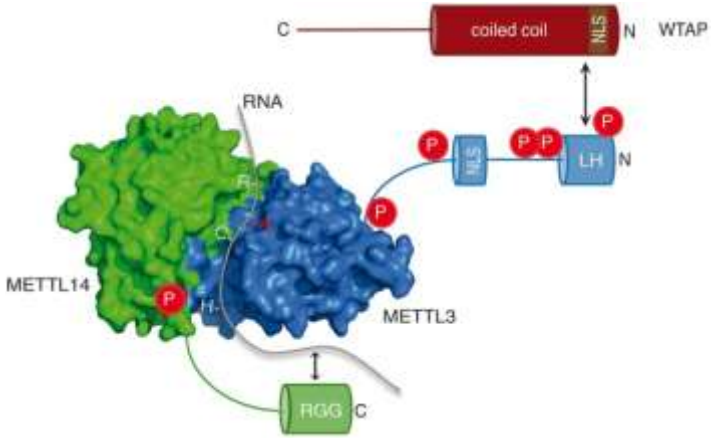


Fig.1.1.2 Core methyltransferase "writer" complex
Schematic representation of the interaction betweenMETTL3 (blue), METTL14 (green), and WTAP (red). METTL3 catalyzes methylation of the adenosine base (red), METTL14 coordinates and stabilizes the RNA binding. (E et al. 2018)

Different other accessory proteins are present that contribute to the dynamics of the methylation.

RNA Binding Motif Protein 15 (RBM15) for instance, helps binding the complex to the correct target while the zinc finger CCCH-type containing 13 (ZC3H13) seems to be important for keeping the whole complex inside the nucleus, as its knockdown causes a rapid translocation of VIRMA, WTAP and HAKAI to the cytoplasm(Wen et al. 2018). The role of HAKAI is not quite well understood yet, but it has been noted that depletion of this protein causes a decrease in the levels of m6A, affects *Drosophila melanogaster* sex determination and *Arabidopsis thaliana* development(Wen et al. 2018). Vir-like m^6A methyltransferase-associated (VIRMA) or KIAA1429 directs methylation to preferential sites. A model was proposed according to which VIRMA serve as a scaffold to hold WTAP/HAKAI/ZC3H13 and binds through WTAP to the MMW complex, in order to guide the deposition of m6A in the 3'UTR and stop codon region(Yue et al. 2018).

The Methyltransferase-like 16 (METTL16) is an independent methyltransferase with a different binding site from the METTL3/METTL14 methylation complex. It seems that it regulates splicing and stability of mRNAs by catalysing m6A in U6 small nucleolar RNA (snoRNA), small nuclear RNA (snRNA) and other long non-coding RNAs (lncRNA) (Warda et al. 2017). Other secondary methyltransferases have been identified, like METTL5 and ZCCHC4 that methylate respectively 18s and 28s rRNA, and many more proteins are reported every year that seem to be involved in m6A, but need to be further explored(Sun et al. 2022).

m6A readers

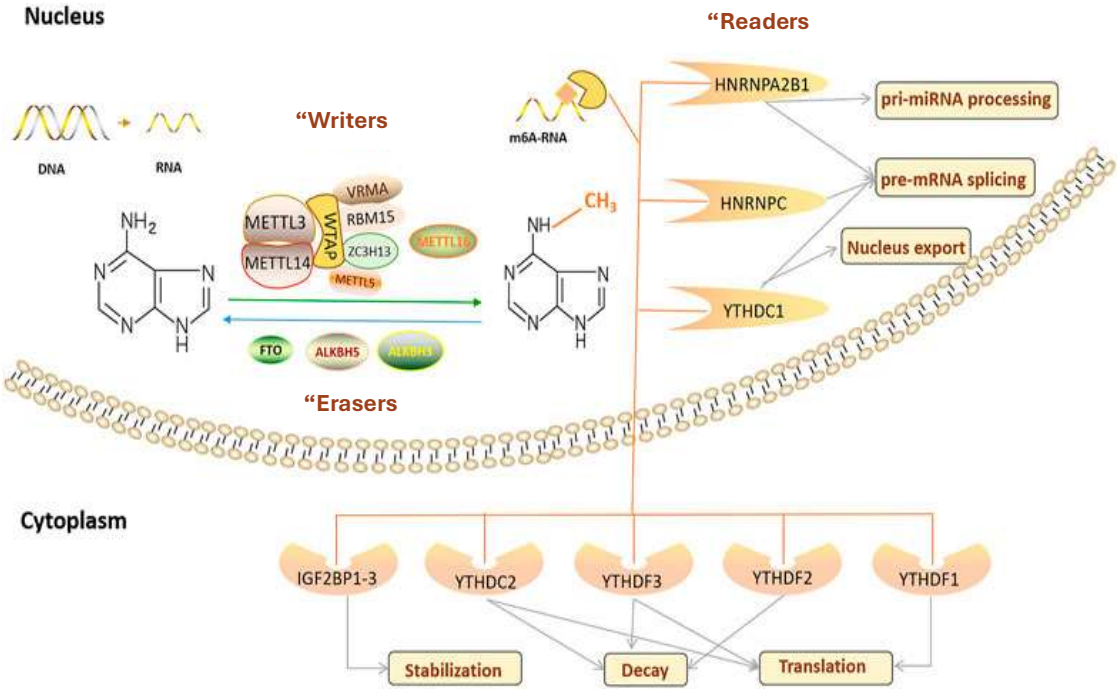
The m6A binding proteins, also called m6A "readers", are deputated to determine the fate of the mRNA, based on the specific location and pattern of the modification. They do this by regulating mRNA stability, splicing, structure, translation efficiency and microRNA biogenesis. Within the readers we can find proteins with YTH domains (YTHDF1/2/3 and YTHDC1/2), heterogeneous ribonucleoproteins including heterogenous nuclear ribonucleoprotein C (HNRNPC), G (HNRNPG) and A2B1 (HNRNPA2B1) and insulin-like growth factor 2 binding proteins (IGF2BPs). Different readers have different cellular localization based on the biological function that they're involved in.
The ones located in the nucleus (YTHDC1, HNRNPA2B1 and HNRNPC) regulate mRNA splicing and miRNA maturation. YTHDF1/2/3, YTHDC2 and IGF2BP1/2/3 are localised in the cytoplasm, where they interact with the translation process, mRNA degradation and mRNA stability(Sun et al. 2022).

m6a erasers

The RNA demethylases or "erasers" can remove the m6a modification. They are localized in nuclear spots where we find also the methyltransferases. The protagonists are the fat mass and obesity-related proteins (FTO), AlkB homologue 5 (ALKBH5) and AlkB homologue 3 (ALKBH3). They're activity is strictly related to the writers, and together they dynamically regulate the deposition of m6A in the transcripts.

Fig. 1.1.3 m6A regulation

Graphic summary of m6A regulation by writers, readers and erasers. (Sun et al. 2022)



1.1.2 m6A in planaria and *Macrostomum lignano*

Planaria, like almost all invertebrates, do not possess the erasers' group. Still, m6A is present and seems to play the same role as in the other species.

Dagan et al. 2022, performed an RNAi experiment in planarian model *Schmidtea mediterranea* against writers and readers, treating the animals in a homeostasis state and after amputation. They observed that RNAi against *kiaa1429*, *mettl14* (writers) and *ythdc-1* (reader) caused hindered regeneration and death in amputated worms. Furthermore, these results were associated with a decrease of m6A methylation, which presence was confirmed on mRNAs. Really similar results were achieved by (Cui et al. 2023), with RNAi on WTAP (writer).

Macrostomum lignano also presents orthologs of writers and readers, but no erasers, and so far, there are no published studies about m6A in this model.

Studying m6A in flatworms, which possess extraordinary regeneration and healing abilities, is fundamental to better understand the mechanisms and dynamics of this processes, as it is better explained in sections 1.2, 1.2.1 and 1.2.2.

1.2 Regeneration

Regeneration is recognized as the process of replacing or restoring damaged or lost cells, tissues or organs. This is possible thanks to the ability of stem cells to proliferate and differentiate. When damage occur, specific signals are captured by stem cells that will start to migrate and proliferate in order to substitute the damaged tissue. This also happens regularly during tissue turnover in a homeostasis state. The extent of regeneration potential is highly variable between phyla. Humans, for example, are only able of limited regeneration, like for a small amount of tissue or a very limited area of an organ, while other organisms, like some planarians, are able of whole-body regeneration. Trying to understand the mechanisms that regulates so differently the same process has been the aim of many studies for years(Gurtner et al. 2008).

Studying regeneration is important for many aspects. First, loss of part or more of a tissue or organ is typical of many diseases and pathological conditions, but most commonly we observe a slowly decline of the correct functioning of cells and tissues during ageing. So, a deep understanding of how regeneration works as much as ageing and loss of function of cells and tissues, is crucial to find a way to contrast diseases and the effects of ageing, and improve the life of living beings, even with these conditions(Tanaka and Reddien 2011).

1.2.1 m6a in stem cells regulation and regeneration

Many important processes like tissue turnover, development and regeneration are possible thanks to the extraordinary renewal abilities of stem cells. We can divide them in two main groups that have different abilities to differentiate, based on the inner differentiation potential that they retain: pluripotent stem cells, like embryonic stem cells or induced pluripotent stem cells (iPSCs), and somatic or nonembryonic stem cells, also called “adult” stem cells. If the first have the ability to differentiate into all types of cells of the adult body, the second ones are usually found in tissues, and can yield into the specific cell type of that tissue. The mechanisms that regulate homeostasis, differentiation and reprogramming of stem cells are broadly studied but so far not completely understood(Miyamoto, Furusawa, and Kaneko 2015).

We know that there are some specific genes necessary for maintaining the pluripotency state or induce differentiation. Genes like *Oct4* and *Nanog*, for example, are expressed during the pluripotent state and deactivated during differentiation.

During normal development, differentiation is irreversible, the sole moment when epigenetic reprogramming physiologically happens is during the fertilization

phase. Interestingly, in 2022 Gill et al. managed to “rejuvenate” fibroblasts using the Yamanaka factors (Sox2, Klf4, Oct4, c-Myc) by reprogramming differentiated cells to a transient pluripotency state and back (Gill et al. 2022).
After these considerations, it is evident how gene expression plays a key role in the regulation of stem cells, hence m6A is most likely to have a play in this.
Indeed, Geula et.al in 2015 observed that a METTL3 knockout is associated with a near-complete depletion of m6a on mRNA and noted that epiblasts and naïve embryonic stem cells (ESCs) failed to terminate their naïve state in a murine model. Even by surviving, these cells were not able to continue the embryonic development.
Later in 2018, Wu et al. conducted a study in murine bone marrow mesenchymal stem cells (MSCs), where they detected impaired bone formation, hindered osteogenic differentiation and osteoporosis after METTL3 knockout. Moreover, overexpression of METTL3 in MSCs protected mice from induced osteoporosis (Wu et al. 2018).
Interestingly, results were noted also in an experiment on rodents’ peripheral nervous system (PNS), where overexpression of the eraser ALKBH5 impaired axonal regeneration, and its knockdown resulted in enhanced sensory axonal regeneration (Wang et al. 2023).
In the last few years, different studies focused on the role of epigenetics in processes like skin wound healing and scar formation. With a focus on m6A, curious findings were made, like how METTL14 upregulates the expression of pvt1, a lncRNA, in epidermal progenitor cells during wound repair, METTL3 regulates the vascular endothelial growth factor (VEGF-C) in lymphatic endothelial cells (LEC) during wound healing and FTO upregulates *colia1* (which express pro-alpha1 chains of type I collagen) in fibroblast during keloid formation(Luo et al. 2023).

It is clear then, how m6A, by being a key component of stem cell regulation, retains a huge importance in better understanding already broadly studied processes regarding tissue turnover, regeneration and development. That’s also why *M. lignano*, by possessing remarkable regeneration abilities, represents an excellent model for studying m6A in stem cells regulation.

1.2.2 Neoblasts

Invertebrates are famous for owning huge regeneration capabilities. Even if not all of them possess this ability, the major part is able of whole-body regeneration as we see in *Schmidtea mediterranea, Dugesia japonica* (Tricladida, Plathyelminthes) and *Hydra* (Cnidaria) or just organ/tissue regeneration as in *Macrostomum lignano* (Platyhelminthes), *Aplysia californica* (Mollusca) and *Alitta virens* (Anellida) (Lai and Aboobaker 2018). This is possible thanks to the presence of adult stem cells called neoblasts. Neoblasts are pluripotent adult stem cells that look like small, round cells with a high nuclear:cytoplasmic ratio and are able to differentiate in any kind of cell in the organism body. The mechanisms by which this happens are still to be completely understood, but some very interesting observations have been made by recent studies. All active neoblasts were found to express the Germline Multipotency Program (GMP) gene *piwi* (or orthologs of *piwi*), that together with *vasa* and *nanos* is part of this conserved set of genes which are important for maintaining multipotency(Lai and Aboobaker 2018). Moreover, in planarians, different classes of neoblast have been identified, the two major groups are zeta (ζ)-neoblasts (epidermal specialized) and sigma (σ)-neoblasts, that give rise to all the other kind of cells. Within the latter group, other sub-groups have been described, the gamma (γ)-neoblasts and nu (ν)-neoblasts. Van Wolfswinkel et al. characterized the sigma class to be able of self-renewal, while zeta neoblasts pass through the M phase just once before terminally differentiate. Nevertheless, the complete characterization of all different classes of neoblast is still far to be completed. Multiple studies also observed that neoblast differentiation involved the expression of fate-specific transcription factors (FSTFs) which are necessary for cell differentiation (King et al. 2024). This was an important discovery, since it allowed the establishment of markers for neoblasts undergoing differentiation. Later, (Raz, Wurtzel, and Reddien 2021) conducted a study on planarians based on which they developed a model where neoblast specialization and fate switching can occur without losing potency. They proposed a non-hierarchical model where specialized neoblasts are able to go through an asymmetric division generating one specialized neoblast and one unspecialized. They, indeed, observed that an epidermal neoblast divided into a post-mitotic epidermal progenitor and to a neoblast that lacked an epidermal fate signature. This means that even specialized neoblast, or at least a sub-population of them, are able to retain pluripotency even after specification. Furthermore, animals treated with irradiation in order to deplete the neoblast population, were able to survive after implantation of single neoblasts that managed to repopulate every kind of differentiated cells.

In the flatworm *Macrostomum lignano* transplantation experiments have not been tried so far, but neoblasts are still at the basis of cell renewal and tissue/organ regeneration. This makes *M. lignano* without doubt a very suitable model to investigate the dynamics of neoblast.

1.3 *Macrostomum lignano*

M. lignano is a free-living marine flatworm belonging to the macrostomorpha clade, the earliest branch of Rhabditophora, firstly found in Lignano, Italy. It lives in the spaces between sand grains in the upper interstitial zone, so it developed the ability to adapt to very different environmental conditions and can survive a broad range of temperatures, salinities and oxygen concentrations. (Wudarski et al. 2020a) It measures around 1-1,5 mm in length and 0.3 mm in width, and we can identify three main parts: head, body and tail plate which are completely covered by a multi-ciliated epidermis. The head region presents two photosensitive eyes between a small area called rostrum, where we also find the brain, on top, and the mouth, underneath. The mouth is connected to the gut through the pharynx and from here to the tail we identify the body, where the gut represents the major area. Aside the gut we can find the gonads: the testis, proximal to the head, and the ovaries, closer to the tail, which are also visible since the animal is transparent (Mouton et al. 2018). *M. lignano* is indeed a non self-fertilizing hermaphrodite, since it possesses both gender's gonads but it needs to interact with another individual in order to reproduce sexually. Between the gonads and the tail, we can find the female opening and in the same region eggs develop after fertilization. The male organ called "stylet" is found on the tail, near the seminal vescicles and above the adhesive glands, located to the end of the tail plate. (Ladurner et al. 2005)

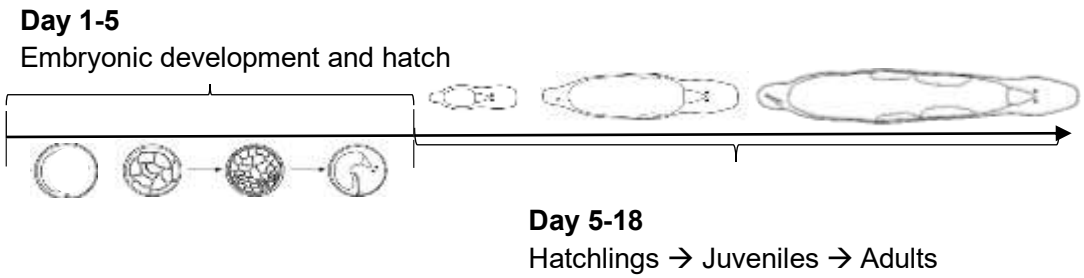


Fig. 1.3.1: Life cycle of *Macrostomum lignano*
The embryonic development of *M. lignano* takes about 3 days at 25°C. After the hatch, the worms are defined "hatchlings" until they reach half the size of an adult and start grow gonads, at that point they're called "juveniles". Then, when all the gonads are formed and they are able to reproduce, they have reached adulthood.

1.3.1 *Macrostomum lignano* as a model organism

By being so small, *M. lignano* can live a quite long amount of time, more than 2 years, and presents high regenerative abilities which makes it a perfect model to study ageing, regeneration and stem cells regulation. Furthermore, culturing *M. lignano* is easy and cheap, the worms are usually kept in petri dishes filled with artificial sea water and fed with unicellular diatom algae of the species *Nitzschia curvilineata*. It can survive between 4°C to 37°C but its ideal temperature is between 20°C-25°C. At this temperature it lays single-cell eggs which develop and hatch within 3 to 5 days.(Wudarski et al. 2020a)

It presents remarkable regenerating abilities, since, if injured it, can regrow from the head every kind of tissue and organs, apart from the brain and the mouth/pharynx region which are needed for the process (Fig. 6). After amputation, for example, it forms a blastema where the incision was performed, which is the region where stem cells proliferate and differentiate to regenerate tissues and organs. (Mouton et al. 2018)

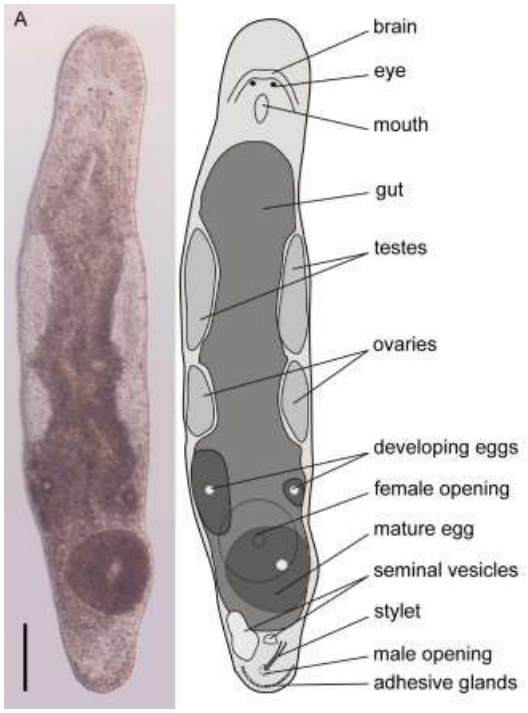


Fig.1.3.2 *M. lignano* morphology Interference contrast photomicrograph and schematic drawing of M. lignano. Scale bar: 100μm (Mouton et al. 2009)

M. lignano is also able to decrease its size when starved, regressing the gonads and reducing the mitotic activity, but it can easily reverse this process as soon as it is fed again. This is possible thanks to the presence of neoblasts. Stem cell population in *M.lignano* is indeed represented by neoblasts (somatic stem cells) that localize on the sides of the worm, and by germline stem cells, present in the gonads (Mouton et al. 2009).

M. lignano presents a genome of 502 Mb (haploid size) and his karyotype consists in two large and six small metacentric chromosomes (2n=8). In addition, studies

revealed that it can survive up to 210 Gy of gamma radiation, which make it also a good model to study genome stability and maintenance.
In the last few years different tools have been adapted for this animal, such as RNA interference and in situ hybridization protocols, transgenesis methods which are possible by microinjection of the eggs, live imaging and fluorescence-activated cell sorting (FACS). Moreover, *M.lignano* is the only flatworm with available transgenesis methods, which makes it even more important as a model organism.(Mouton et al. 2018)
Finally, data about the genome and transcriptome sequencing are available at https://gb.macgenome.org/ and https://sc455.macgenome.org/(Wudarski et al. 2020b).

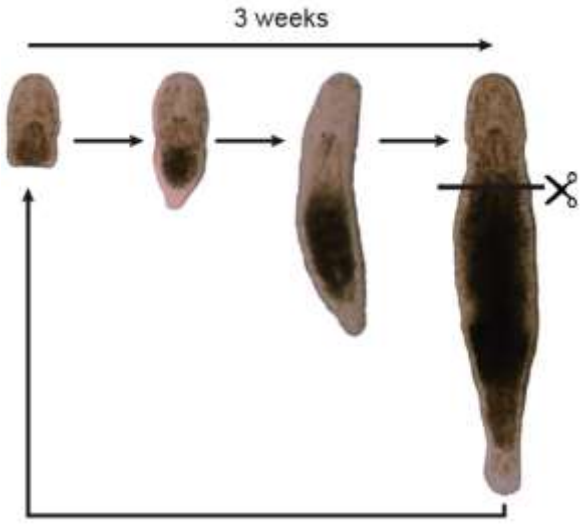


Fig. 1.3.3 Regeneration time of *M.lignano* after amputation
After cutting underneath the head, a blastema is formed and the worms regenerate completely in 3 weeks.

1.3.2 *Macrostomum lignano* and m6A

Considering the availability of numerous novel methods to study *M.lignano* biology, it is now more clear why this model organism is ideal for research on m6A methylation. It's ease of culture and rapid reproduction allow efficient experimentation, while its remarkable longevity and regenerative abilities makes it a perfect suit for studying epitranscriptomic regulation of cellular maintenance and stem cells function, which is possible thanks to the availability of well-established techniques.

Chapter 2: Aim of the project

The role of m6A methylation in different biological processes is increasingly recognized. However, deeper investigations performed in the last years revealed how much complex and intricated is the regulatory network underlying this modification.

This is why using a novel, yet to be fully explored, model organism such as *Macrostomum lignano,* which retains the characteristics to study many processes where m6A is known to play a key role (stem cell regulation, regeneration, ageing) is necessary if we want to achieve better knowledge on this modification.

In particular, this research project was inspired by some novel results observed from studies on the planaria model *Schmidtea mediterranea,* which is evolutionary closer to *M. lignano* than the other model organisms used to study m6A. *S. mediterranea* possesses the ability of whole-body regeneration thanks to the activity of neoblast stem cells. Different studies (Cui et al. 2023; Dagan et al. 2022) observed how m6A methylation is fundamental for neoblasts in planaria to carry out their function. Moreover, there are not published studies yet about m6A in *Macrostomum lignano,* which makes the aim of this project even more important.

This specific study started from some previous results obtained a few years ago by a master student (Dana Frank) in the same laboratory. She performed a homology search with bona fide gene sequences of the m6A regulatory proteins from human and planarian assemblies and identified the presence in *M. lignano* of at least one homolog of the writers and readers genes, except for YTHDF1 (reader) which, together with the erasers' genes, is not present. She also carried out RNA interference (RNAi) and in situ hybridization (ISH) experiments on adults and amputated worms, to visualize the pattern of expression of the genes in the worms physiologically and after RNAi treatment. However, the techniques for RNAi and ISH that she used were still not fully optimized at the time of her project.

So, the first part of my project focused on repeating the experiments using more established protocols for RNAi (Mouton et al. 2024) and ISH (Ustyantsev et al. 2024) and observe if the previous results were confirmed or not, with a focus on the writers' genes. We then wanted to investigate the role of those genes in different life stages of the worms, performing RNAi on eggs and hatchlings/juveniles (from the hatch of the egg to adulthood).

We can therefore categorize the aims of this project as the following:

1. Repeat experiments already performed, with more established tools and confront and/or validate the previous results.
2. Investigate the consequences of gene knock-down for the proteins involved in the deposition of m6A in different phases of the life of the model organism.
3. Lay the foundation for future investigations in this matter.

Chapter 3: Materials and methods

3.1 Culturing Macrostomum lignano

M. lignano is cultured in Petri dishes containing 32‰ Artificial Sea Water (ASW), grow in incubators with 60% humidity at 20/25°C that keep a 13/11h light/dark cycle (Mouton et al. 2018). Animals are fed with the diatom *Nitzschia curvilineata* (Bacillariophyceae) which grows in the f/2 medium, consisting of vitamin enriched ASW. Before transferring diatoms to a worms' culture, f/2 medium is changed with ASW to avoid overgrow of the algae, which can results is producing domoic acids that it's toxic for worms (Wudarski et al. 2020b).

Worms are usually fed once a week, to avoid starvation, but different cultures may need a diverse management. Cultures used for eggs microinjections, also called "Injection cultures", are bigger than a normal one and can contain up to 1000 worms or more, which are synchronized to be at the same age. It has been indeed observed that in this condition worms are more prone to lay eggs, so these specific cultures are transferred twice a week to avoid contamination from hatchlings(Wudarski et al. 2017).

For this project I used two specific lines of worms:

- NL12S23: wild-type worms from the inbred line NL12 that were checked in 2023 (-S23) for chromosome duplication. This line was used for ISH experiments and are the same present in the injection cultures.
- NL45: this line was created by knock-in of the fluorescent protein mNeonGreen into the H2AX histone gene, a neoblast marker. This fusion protein is localized in the nucleus of neoblasts, so the more intense fluorescent signal comes from these cells, but even during differentiation the protein is retained inside the nucleus until degradation, and because of this the signal fades from the neoblast populated areas on the sides of the worm, to the centre, where more differentiated cells are. This transgenic line is ideal to study neoblast differentiation and proliferation, since the signal and its position are related to the activity of neoblasts, and is hence been used for the RNAi knockdown experiments.

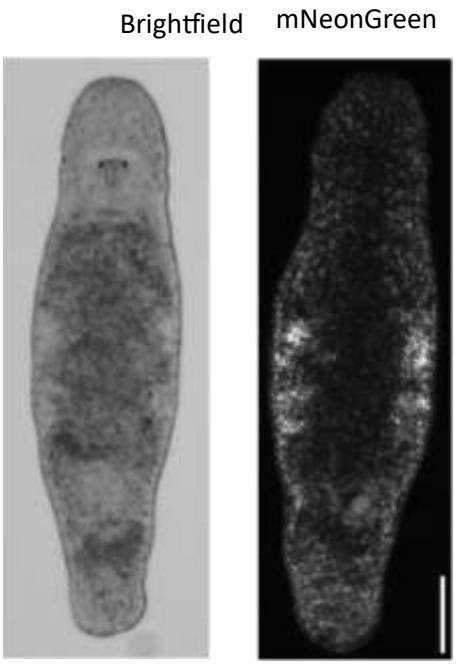


Fig. 3.1.1: NL12S23 and NL45 transgenic lines

(A) NL12S23 worm: this is a wild-type adult. Due to its transparent nature under a normal light are visible the internal parts like gonads (on the side with a round shape), eyes, the mouth underneath, a lighter shape upon the eyes represents the brain and the darker region in the centre is the gut

(B) NL45 worm: more intense signal from lateral bands and gonads indicates proliferative cells, this intensity it's lost with cells undergoing differentiation. The green signal is showed here in white to be better visualized.
Scale bar: $100\mu m$

3.3 RNA interference

3.3.1 Molecular mechanism and limits of RNA interference

RNA interference is a well establish method for gene knockdown. Its molecular mechanisms require a dsRNA molecule that, once in the cytosol, is processed by a riboendonuclase called DICER, which is able to cleave the dsRNA molecule into smaller 20-25 bp fragments called short-interfering RNA (siRNA). These fragments are then recognized by the RISC complex, able to identify the mRNA which sequence is complementary to the respective siRNA and degrade it, hence preventing protein production. Another important role for the efficacy of RNA interference is played by the RNA-dependent RNA polymerase (RdRp), which activity is responsible of the exponential creation of secondary siRNA when these are associated to the mRNA, enhancing the effect of knockdown (Ranasinghe et al. 2023).

Despite it has been proven to be an excellent knockdown technique, RNAi presents its limits. First of all, the efficacy of this method is directly related to the gene expression, indeed, as I already specified, more siRNA are produced when bonded to the mRNA molecule, so if the transcription rate of the gene of interest is low, RNAi efficacy is limited.

Secondly, there is always the possibility of off-target effects, which happens when the RISC complex associates the siRNA with a different molecule than the targeted one, usually because of sequence similarity. A recent study showed that dsRNA with >80% sequence identity are enough to trigger RNA efficiently as much as dsRNA with >16b of perfectly matched sequence or >26 bp of almost perfect match (Chen et al., 2021). So, when choosing the sequence to match the mRNA, is fundamental to check the specificity and length of the dsRNA. Furthermore, the delivery system of dsRNA plays an important role in obtaining good results. In *M.lignano,* for example, the first RNAi protocol included soaking the worms in wells with diatoms (Pfister et al. 2008), but lately it was realized that diatoms had an inhibitory effect on RNAi since they present a shell made of hydrated silicate which is known to be able to immobilize nucleic acids. To prevent this, the new protocol indeed introduced soaking during a day of starvation. (Mouton et al. 2024).

3.3.2 dsRNA production for RNA interference in *M.lignano*

Plasmid creation

After individuating a 400-600bp target region of the gene of interest, the sequence is PCR amplified starting from cDNA using the proof-reading DNA polymerase KAPA (Roche) mix. (Table 3.2.1 and 3.2.2)

Table 3.2.1: PCR amplification reagents

Reagent	Volume (μL)
cDNA	1
Primers (fwd+rev, 10 μM)	1,25 each
H_2O	9
2X KAPA hifi hotstart ready mix	12.5

Table 3.2.2: PCR program

Phase	Temperature	Time	N°cycles
Initial denaturation	95°C	5’	
Denaturation	98°C	15’’	30
Annealing	60°C	15’’	
Elongation	72°C	30’’	
Final elongation	72°C	2’	

The PCR product is then loaded into a 1% agarose gel stained with SYBR-SAFE and 5 μL of NEB 6x purple loading dye, then run for 30’ at 80V. Once the product is confirmed to have the correct size, it is purified from the gel by Freeze&Squeeze (freeze at -20° then centrifuge at maximum speed for 3 minutes).

The T444T plasmid vector used for the cloning is then cut separately by KpnI and BglII restriction enzymes, then purified with the aid of Qiagen PCR clean up protocol.

Table 3.3.3 Mix KpnI. Reaction lasted for 15' at 37°C.

Reagent	Volume(μL)
T444T vector	26
10XCutSmart	3
KpnI enzyme	1

Table 3.3.4: Mix BglII. Reaction lasted for 1h at 57°C.

Reagent	Volume (μL)
Mix KpnI	30
BglII enzyme	3
10X 3.1 buffer	10
H_2O	57

The next step involves the Gibson assembly procedure to insert the amplified sequence into the vector. The reaction mix is added to a 200 μL tube with a drop of halocarbon oil to prevent evaporation. The reaction is carried in a thermocycler at 50°C for 30-60min (Table 3.3.5).

Table 3.3.5: Gibson assembly

Reagent	Volume(μL)
2XNEBuilder HiFi MasterMix	1
BglII and KpnI digested and gel purified T444T plasmid	0,5
Gel purified PCR product	0,5

The reaction solution is then transferred into a 1,5mL tube where competent cells were subsequently added.

Plasmid transformation into competent cells and glycerol stocks

The purified plasmid is then transformed in competent HT115 *E. coli* cells.
Competent cells are defrosted from -80°C in ice, then added in the same tube of the purified plasmid where they undergo transformation by heat shock procedure. The sample is incubated 30' in ice and 45'' at 42°C, then on ice again for a few seconds. Under flame, 900 μL of LB are added then incubated at 37°C for 1h.
After spinning the tube at 4000g for 1-2 minutes, 800 μL are discarded, and the remaining 100 μL are resuspended and plated on an LB+2XAMP agar plate, then left to grow overnight.

The next day, 8 random bacterial colonies are picked from the plate to perform colony PCR and verify the correct integration of the plasmid into the cells. Each colony is resuspended in 15μL of milliQ H_2O in a 200 μL PCR tube.
For the amplification was used the #375_Universal_T444T_T7_FWD primer (TTATGCTAGTAATACGACTCACTATAGGG). The thermocycler program is described in Table 3.3.7

1 μL for each of the colony mix was added to 9 μL of the MasterMix used for the amplification (Table 3.3.6)
PCR products are then checked on 1% agarose gel with EtBr running for 30' at 80V.

Table 3.3.6: Colony PCR reagents

Reagent	Volume(μL)/sample
2X OneTaq Mix	5
#375_Universal_T444T_T7_FWD	1
H_2O	3

Table 3.3.7: Colony PCR program

Temperature	Time	N° of cycles
94°C	5'	1 cycle
94°C	10''	20 cycles
58°C	10''	
68°C	45''	
68°C	1'	1 cycle

The bacterial sample with the correct plasmid is then grown overnight in 4mL of LB+2XAmp. The next day, 0,5 mL of the grown culture is mixed with 0,5 mL of 50% glycerol, then stocked at -80°C.
Part of the same culture is used for MiniPrep and sent to be sequenced.

dsRNA production from HT115 *E.coli* stock cultures
Glycerol stocks are taken from the -80°C freezer and scraped under a flame using a flame-sterilized microbiological loop, then the bacteria are inoculated in 4mL in fresh liquid LB+AMP medium. Cells are left to grow overnight shaking at 180-200 rpm at 37°C. The next morning, the tubes are centrifuged at 4000g for 5min, the supernatant removed, and the pellet resuspended in 4mL of fresh LB+AMP. 200 μL of the night cultures are then added to 20mL of fresh LB+Amp, the tube closed with aluminium foil and the cultures grown for 3h at 37°, shaking at 200rpm.

Next, 100 μL of 0,1M IPTG are added for each culture and continue the growth in the same conditions for additional 4,5-5h.
Upon growth completion, tubes are closed with the original lids and centrifuged at 4000g for 7'. Supernatant is discarded and the samples kept in freezer at -20°C.

3.3.3 dsRNA extraction

Step 1: Nucleic acid extraction

For this step the following reagents are needed:

- Lysis buffer (4% SDS, 0,5M NaCl in MilliQ H_2O)
- 5M NaCl in MilliQ H_2O
- 70%EtOH in MilliQ H_2O
- 100% isopropanol

LB buffer is preheated at 60-80°C to dissolve SDS, then 665 μL are added to the defrosted cell pellet to resuspend by pipetting. 665 μL of the lysate are transferred in 2mL Eppendorf which are then left in the oven at 80°C for 10'.
335 μL of 5M NaCl are added to the lysate and mixed by shaking/inverting the tube to precipitate SDS. Then centrifuge at 4°C, for 10' at maximum speed (18-21kg). Transfer the supernatant in a new 2mL tube, minimizing carry over of the pellet.
950 μL (1 volume) of 100% isopropanol is added and mixed by vigorous shaking/inverting the tube and centrifuge at maximum speed for 15', then decant the liquid. To wash away any SDS residues, 1mL of 70-75% of EtOH is added, then the tubes are inverted to wash the walls, centrifuged at maximum speed for 5' and the liquid is decanted. This step is then repeated another time. This wash step is followed by air-drying for 10-15'. The pellet is then resuspended in 450 μL of Nuclease-free water then transferred into a new 1,5 mL tube.
To get rid of every kind of nucleic acid but dsRNA, DNAse I and RNAse T1 are used. After adding 50 μL of 10X DNAse buffer, 2 μL of each enzyme are mixed, then incubated at 37°C for 30'.

Step 2: removal of *E. coli* 5S dsRNA fraction

For this step, the following reagents need to be prepared:

- 50% PEG8000 in MIlliQ H_2O (w/v)
- 5MNaCl in MilliQ H_2O
- 70% EtOH in MilliQ H_2O
- RNAse free H_2O

The samples are then centrifuged at full speed for 5-10’ to pellet any residual SDS flakes. The supernatant is then transferred into a new 1,5 mL tube where 74 μL of NaCl and 166 μL of 50% PEG8000 are added. Mix everything by vigorously by inverting the tube until homogeneous. The mixture is then left sit at room temperature for 15-20’, then centrifuged at maximum speed for at least 20’.
Next, after discarding the supernatant, 500 μL of 70% EtOH are added to wash away the salt and PEG. After 5’ of centrifuge at maximum speed and the supernatant discarded, repeat the wash step and leave the samples to air-dry for 10’. The pellet is finally resuspended in 200 μL of Nuclease-free water.

Step 3: agarose gel dsRNA integrity analysis and dsRNA concentration estimation
Sample integrity is checked by gel run in 1% agarose gel with EtBr. 1 μL of each sample is added to 9 μL of MilliQ and 2 μL of 6X Gel Loading Dye, then loaded in the gel and run at 80V for 30’.
Sample quantification is achieved by densitometric analysis of the picture of the gel bands, using ImageJ.
Once quantified, the samples were divided into 10-15 μL aliquots containing 2μg of dsRNA, then used for the RNAi experiments.

3.3.4 RNAi by soaking

The following RNAi by soaking protocol is based on the one described on Mouton et al. 2024. Worms from the NL45 transgenic line were selected and divided ≈40 individuals per well, they were then starved 24h before starting the treatment. Twice a week (on Mondays and Thursdays) worms are transferred in a 24 well plate, each well filled with 300 μL of ASW, where the dsRNA solution is added.
The event of adding the dsRNA solution to the worms will be referred as “soaking”. dsRNA aliquots are kept at -20°C and defrost right before using them, each aliquot contains 2 μg of dsRNA in a maximum volume of 15 μL.
After 24h of soaking, worms are transferred in a 12 well plate filled with 0,7-1 mL of ASW and seeded with diatoms. They are fed until the next soaking, and kept at 25°C. Before transferring the worms in a new 24 well plate for treatment, they are washed in ASW to minimize diatoms carryover.

3.3.5 Amputations

To perform amputations, worms need to be immobilized. In order to do that, the animals are put in a drop of $MgCl_2$ hexahydrate and once stabilized they are transversally cut in the region between the pharynx and the gonads with a scalpel. This operation can last for not much more than 10-15 minutes otherwise the worms can get damaged interfering with the observation for a phenotype and in the worst cases they can die.

3.3.6 Imaging

Images were taken with the Zeiss Axio Zoom V.16 stereo fluorescent microscope using the MRc5 color 307 camera for ISH pictures and AxioCam HRm CCD camera for fluorescent pictures.
Live imagine was performed by selecting the worms, immobilizing them in a drop of $MgCl_2$ hexahydrate then aspirating as much liquid as possible to flatten the worms so that every part of the body is well recognizable.

3.3.7 Microinjections

The protocol for microinjections for *M.lignano* eggs was developed a few years ago by Wudarski et al. 2017 and proved efficient in many transgenesis experiments.

Eggs picking

One or more injection cultures are starved for the night, the next morning they are transferred in a new Petri dish filled with ASW and then left in a dark place for 2-3h. When put in the light again they start laying the eggs, usually on the margins of the dish.
To pick the eggs a glass capillary with a toothbrush bristle glued on the extremity is used. The eggs are picked from the petri and put in a row on a slide with some ASW. If the microinjecting procedure could not be performed right away, eggs were kept in the fridge at 4°C to slow down development.

Preparation of needle and holder

The aluminum silicate needle is created with the Sutter P-1000 micropipette puller. One of the extremities is heated up and then stretched. The same procedure is needed for the holder, but the latter will have the extremity bent, in order to have a bigger and smoother end. Needle and holder are then positioned respectively in the TransferMan NK2 and PatchMan NP2 arms which are manually controlled.

Injecting

An AxioVert A1 inverted microscope (Carl Zeiss, Germany) was used to perform all of the micromanipulations, while the FemtoJet express (Eppendorf, Germany), was used as the pressure source for microinjections.

Before injecting, the needle extremity that has been prepared needs to be broken. This is done by pushing the needle against the holder until it breaks.

Before inserting the construct, the tube with the sample was centrifuged at maximum speed for 3' to push on the bottom dust or particles that could clog the needle.

The injection follows this procedure:

1. Approach a cell with the holder and put a little pressure on it.
2. Slowly insert the needle into the cell, until you can see that the external membrane has been broken but be careful not to damage too much the cell, otherwise it will die.
3. Inject the construct and take the needle out of the cell.
4. Proceed with the next cell.

After the procedure, slides are put in 6 well plates filled with ASW and kept at 20 - 25°C.

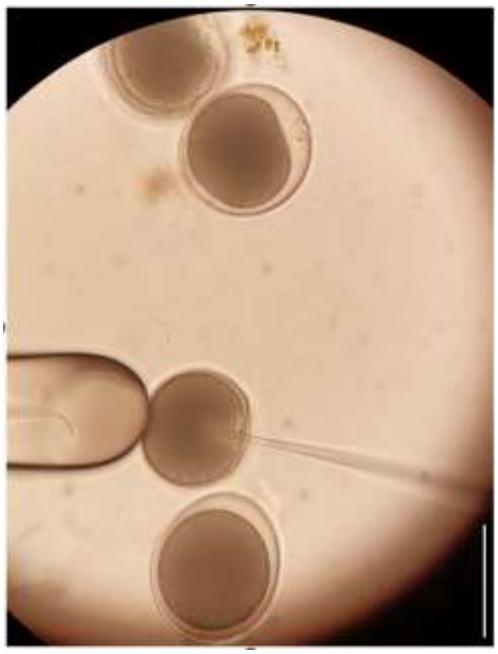


Fig. 3.7.1: Microinjection procedure

On the left, the holder is slightly pushing the egg while the needle has penetrated the eggshell and is ready to inject.

Scale bar: 100µm

3.4 SABER-ISH

In situ hybridization: molecular principles

In Situ hybridization (ISH) is a well-established technique used to localize a particular sequence of a nucleic acid. This is possible by creating specific probes complementary to the molecule we are interested in and characterized by a fluorescent (FISH) or chromogen (CISH) labelling. This method is broadly used in pathology studies, for example, when looking for chromosome abnormalities or expression anomalies but finds application in many other fields such as microbiology, developmental biology, karyotyping and phylogenetic analysis (Gall 2016). For this project, I used a newly developed protocol specifically adapted for *M.lignano* from Ustyantsev et al. 2024, which developed the OneSABER platform connecting multiple ISH approaches. Between all the methods, I used the AP-SABER ISH (AP: alkaline phosphatase) through which I aimed to target the mRNA of the writers' genes, to visualize their expression pattern.

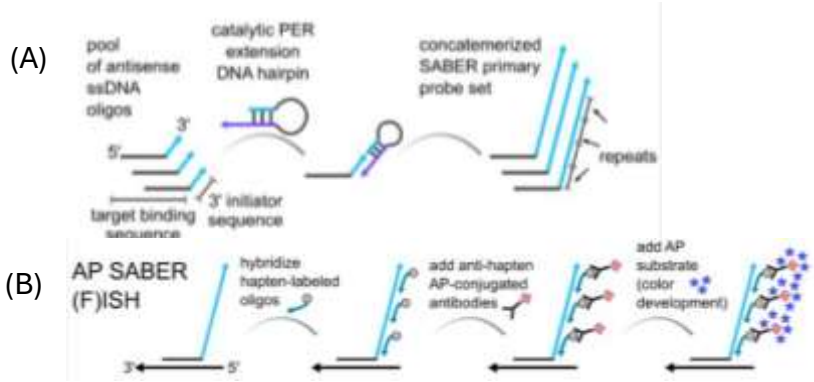
AP-SABER ISH: molecular mechanisms

The primary probe presents the sequence complementary to the mRNA of interest. It's created by combining together a pool of 15-30 custom user-defined short oligonucleotides (35-45 nt), presenting at the 3' a 9nt initiator sequence which is extended *in vitro* through a Primer Exchange Reaction (PER). PER operates with a catalytic DNA hairpin which, by binding the 3' initiator sequence, allows the concatenation of small oligonucleotides that will be recognized by the secondary probes. The latter are small ssDNA adapters that can change according to the chosen signal development method. In my case, I used secondary probes conjugated Digoxigenin, that are then recognized by Anti-Dig antibodies in turn bond to the alkaline phosphatase. Finally, by adding the AP substrate Vector Blue (Vector Labs) a blue signal develops showing the physical expression pattern of the genes.

Fig. 3.4.1: SABER-ISH molecular mechanisms. (Ustyantsev et al. 2024)

(A) Primary probe creation.

(B) AP SABER ISH



3.4.1 SABER-ISH protocol

The recipes and protocol have been adapted from Ustyantsev et al. 2024.

Probes creation

Probes were ordered as desalted dry oligonucleotides which were then diluted in 1XPBS to reach a final concentration of 10µM. The SABER reaction reagents are described in Table 4.1.1.

Table 4.1.1: SABER reaction reagents

Reagent	Volume (µL)
10XPBS	10
100mM MgSO4	10
dNTP mix (A,T,C) 6mM	5
Clean G oligonucleotide 1µM	10
PER Hairpin (5µM)	10
Bst DNA Polymerase (8U/µL)	2,5
Milli-Q H_2O	42,5
Probe oligo pool (10 µM)	10

Each component was added to the mix in ice, except for the Bst polimerase and the oligo-pool which were added after 15' at 37°C. The reaction continued for 1h at 37°C and was stopped by raising the temperature at 80°C for 20'.

Each sample size was then assessed by running a 1% EtBr agarose gel, but, since ssDNA would be hardly visible on the gel, 1 µL of the created probe was added to 1 µL of a matching antisense oligonucleotide, to permit the dye to intercalate between the double strands. Gel was run for 30' at 80V. My probes lenght was between 500-900bp.

The rest of the probes was purified by the Qiagen PCR clean up protocol and concentration assessed by Nanodrop. Stock solutions were kept at -20°C and defrosted as needed.

Solutions receipts

→Stock solutions

7.14% $MgCl2*6H2O$ (100 mL):

Dissolve 7.14 g of $MgCl_2*6H2O$ in Mili-Q water (adjust the volume to 100 mL). Pass through 0.22-0.45 µm filter;

1xPBSTw (50 mL):

- 5 mL of 10xPBS (calcium and magnesium-free);
- 500 µL of 10% Tween 20 (0.1%);
- Mili-Q water up to 50 mL;

2xSSCTw (50 mL):

- 5 mL of 20xSSC;
- 500 µL of 10% Tween 20 (0.1%);
- Mili-Q water up to 50 mL;

WashHyb (500 mL, store in 15 mL tubes at -20°C, consumption 3 mL per column):

- 50 mL of 20xSSC (2x);
- 200 mL of 100% deionized formamide (40%);
- 25 mL of 10% Tween 20 (0.5%);
- 225 mL of Mili-Q water;

PreHyb (150 mL, store in 2 mL tubes at -20oC, consumption 250 µL per column):

- 15 mL of 20xSSC (2x);
- 60 mL of 100% deionized formamide (40%);
- 3 mL of 50xDenhard's solution (1x);
- 3 mL of 10 mg/mL salmon sperm DNA (200 µg/mL) (1x);
- 1.5 mL of 10 mg/mL Heparin (100 µg/mL);
- 15 mL of 10% Tween 20 (1%);
- 52.5 mL of Mili-Q water;

Hyb (100 mL, store in 2 mL tubes at -20oC, consumption 170 µL per column):

- 10 mL of 20xSSC (2x);
- 40 mL of 100% deionized formamide (40%);
- 2 mL of 50xDenhard's solution (1x);
- 2 mL of 10 mg/mL salmon sperm DNA (200 µg/mL) (1x);
- 1 mL of 10 mg/mL Heparin (100 µg/mL);
- 10 mL of 10% Tween 20 (1%);
- 20 mL of 50% (w/v) Dextran sulphate in Mili-Q water (10%);
- 20 mL of Mili-Q water;

VectorBlue buffer (10 mL):
• 9.9 mL of 0.1 M Tris-HCl (pH 8.5);
• 100 µL of 10% Tween 20 (0.1%);

Glycerol mounting solution (50 mL):
• Weigh 31.7 g of 100% glycerol (80%);
• 50 µL of 10% Tween 20 (0.01%);
• Mili-Q water to 50 mL;

→General solutions
4% formaldehyde fixation solution (2 mL):
• 267 µL of 30% formaldehyde (MeOH-free);
• 1733 µL of 1xPBS (no Tween!!!);

4% formaldehyde re-fixing solution (1 mL):
• 134 µL of 30% formaldehyde (MeOH-free);
• 866 µL of 1xPBSTw;

ProteinaseK permeabilization solution (2 mL):
• 2 µL of 20 mg/mL proteinaseK stock solution (1/1000 dilution, stored at –20oC);
• 2 mL of PBSTw;

H_2O_2/Formamide Bleaching and permeabilization solution (2 mL):
• 1750 µL of Mili-Q water;
• 100 µL of 100% Deionized Formamide (5%, stored at –20oC);
• 50 µL of 20xSSC (0.5x);
• 80 µL of 30% H2O2 (1.2%);
• 20 µL of 10% Tween 20 (0.1%);

→Alkaline phosphatase colorimetric ISH development substrate solutions
VectorBlue development solution (2.5 mL):
• 2.5 mL of VectorBlue buffer (prepared in advance);
• 1 drop (~40 µL) of VectorBlue Reagent 1;
• 1 drop (~40 µL) of VectorBlue Reagent 2;
• 1 drop (~22.5 µL) of Reagent 3;
Add Reagents 1-3 sequentially, mixing well in-between.

Day 0: Fixation and 1st permeabilization

Worms are starved 24-48h.
They are then moved into a small Petri dish lid, where they are collected with the smallest volume possible and transferred in the plate of the small Petri, then immobilized by adding 3mL of 7.14% $MgCl_2$*6H2O. After eliminating as much liquid as possible, 2mL of 4% formaldehyde fixing solution are added quickly, followed by 200 µL of 100% glacial acetic acid. After 25 minutes of fixation 2 µL of 10% Tween20 are added, which helps the worms to not stick together or in the pipette tip. After 30 minutes in total of fixation, worms are transferred in a 2mL Eppendorf tube and 4x5min washes with 500 µL of PBSTw are performed.

Dehydration and storage
To conclude the washing step, one buffer exchange and one 5 minutes wash with 50% MeOH/PBSTw are performed ad then repeated with 100% MeOH.
Worms then can be kept at -20°C for several months, or, after 30 minutes of dehydration, can be used for hybridization on the same day.

Day 1: Permeabilization and hybridization of primary SABER probes

Rehydration
If warms were kept at -20°C, the solution is changed with room temperature 100% MeOH and 1x5min wash with 50% MeOH/PBSTw is performed, followed by a final 3x5min wash with PBSTw.

2nd permeabilization
Before use, ProteinaseK solution is preheated to 37°C, then 500 µL of it are exchanged with PBSTw and the tubes kept inside the oven at the same temperature for 8 minutes if treating amputated worms or 14 minutes for adult worms. One minute before the end of the incubation time, ProteinaseK solution is discarded, and the worms are briefly washed with 1mL of PBSTw.
PBSTw is then exchanged with 500 µL of 4% formaldehyde re-fixing solution which is incubated for 15 minutes. After the second fixation, the solution is removed and 3x5min washes with SSCTw are performed.
SSCTw is then substituted by 2mL of H_2O_2/Formamide Bleaching solution, which needs to be incubated for at least 45min to 1h under direct LED light illumination. At the end of the bleaching phase, worms are washed with 3x5min washes in 2xSSCTw.
In the meantime, hybridization columns are assembled and preHyb, Hyb and WashHyb brought from -20°C to room temperature.
Pre-hybridization and hybridization

After bleaching, worms are transferred in a small petri dish filled with 3mL of 2xSSCTw to get washed and be selected before being transferred to hybridization columns, prefilled with 400 µL of 2xSSCTw. One buffer exchange and 2x5 min washes with WashHyb are performed, before transferring the columns inside the oven, preheated at 55°C.
WashHyb is then exchanged to 250 µL/columns of preHyb and incubated for at least 30min. In the meantime, SABER probe hybridization solution is prepared by adding 1-1.5 µL of dedicated SABER probe in 170 µL of Hyb buffer. After the pre-hybridization time, the hybridization solution is added for each column, and left overnight at 48°C.

Day 2: Post-hybridization washes and hybridization of secondary probes

Post-hybridization washes
The next day, 250 µL WashHyb are added on top of the Hyb solution, then slowly flushed down. Afterward, 2x30min washes with 400 WashHyb are performed at 48°C, followed by 2x5min washes with 2xSSCTw.
The oven is then cooled at 42°C and the 2x5min washes with preheated PBSTw are performed.

Secondary probes hybridization
Secondary probe solution made of 1-1.5 µL of 10 µL secondary probe and 120 µL of PBSTw is added to each column, then incubated for 1h at 42°C. To follow, 3x5min washes with PBSTw are required.
At this point, columns are transferred at room temperature.

Anti-hapten antibody blocking and incubation
The antibody solution is prepared by mixing the antibody, in my case the alkaline phosphatase, in PBSTw with a 1:4000 dilution factor. Worms are incubated with 250 µL of the antibody solution for 45 minutes at 37°C. The incubation is followed by 1x5 min, 10 min, 15min and 30 min PBSTw washes.

Day3: Signal development and sample mounting

Signal development

The following day, 1x5min incubation with 400 µL of the Vector blue detection buffer is needed, before transferring the worms to a 24 well plate, filled with 300 µL of the detection buffer.

Before adding the development solution, as much detection buffer as possible is aspirated, and at least 400 µL of the color development solution are added.

The time needed to develop a good and clear signal is variable, based on how much the gene is expressed and the signal development used. In my case, all gene developed in ≈2,5h.

To stop the reaction, worms are transferred twice in 1mL of MilliQ water with 0,01% Tween20.

Worms are finally transferred in wells pre-filled with glycerol mounting solution.

Mounting slides

The mounting step is done with the assistance of a stereomicroscope.

A maximum of 4 worms are collected and positioned on the top of an ethanol-cleaned glass slide. Then, using a plastic pipette tip, some Vaseline is picked and adjusted at the corners of a rectangle corresponding to the size of the coverslip that will be added by gently pressing it down with two pipette tips, until the desired worms positioning is achieved.

Chapter 4: Results

4.1: RNA in situ hybridization: characterization of m6A writer's genes expression pattern in adult and regenerating worms

Genes expression patterns were visualized and confirmed by SABER-ISH on RNA, on NL12 worms, following the newly developed protocol by Ustyantsev et al. 2024, as described in the methods section.

Precedent sequencing analysis characterized the genomic and transcriptomic profiles of *M.lignano*, and are available at the websites specified in section 1.3.1. The single cell atlas and RNA tomography already gave me an idea of where to map the expression of the genes in the worms, but an ISH experiment was needed to have a visual confirmation and to prove the efficacy of RNA interference (Fig.: 4.1.1). All writer's genes are mainly expressed in proliferating cells, so we can visualize them on the lateral bands where neoblasts reside and in the gonads, in homeostasis adult worms, while in the amputated ones we can appreciate the blastema formation. (Fig.: 4.1.4-5)

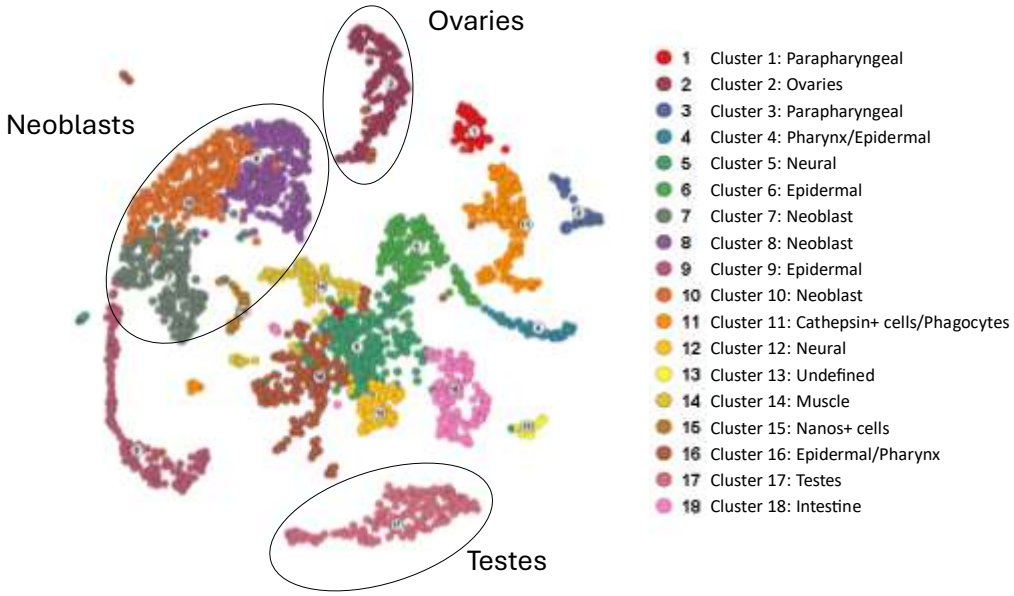


Fig. 4.1.1: Single cell clusters of *M.lignano*

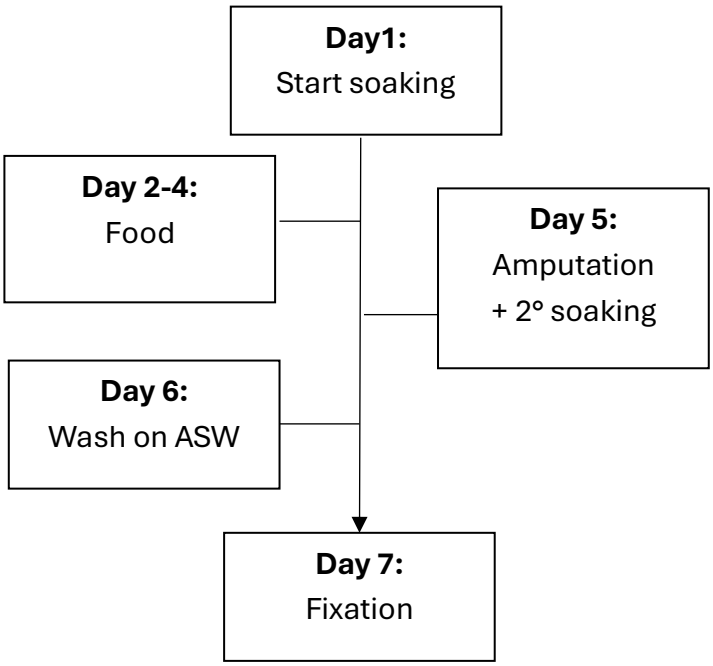
Cells were grouped based on similar gene expressions; I circled the clusters where m6A writers are expressed. Single cell atlas specific for m6A writer's genes are present in the supplementary materials Fig. 1. (https://sc455.macgenome.org/varid_clusters)

This same experiment was performed also by the previous student who analysed the role of m6A writers, but since then, the ISH protocol has been greatly updated and the experiment needed to be re-validated.

For each gene to analyse I associated a positive control (*h2ax*) and a negative control (*heh1*). Each condition had both samples of homeostasis and amputated worms. The worms treated with RNAi with the negative control were used to visualize the gene pattern, while worms treated with RNAi against the gene of interest were used to confirm the efficacy of RNAi. Worms for the positive control were untreated and used as biological and technical control.

Considering day 1 as the first day of soaking for the RNAi treatment, at day 5 I performed the amputation and the second soaking, then the next day I transferred the worms a in 12 well plate filled with 1 mL of ASW, to help get rid of most of the diatoms that could still be inside the worms. At day 7 I fixed them. (Fig.: 4.1.2)

Fig. 4.1.2: Timeline of the experiment



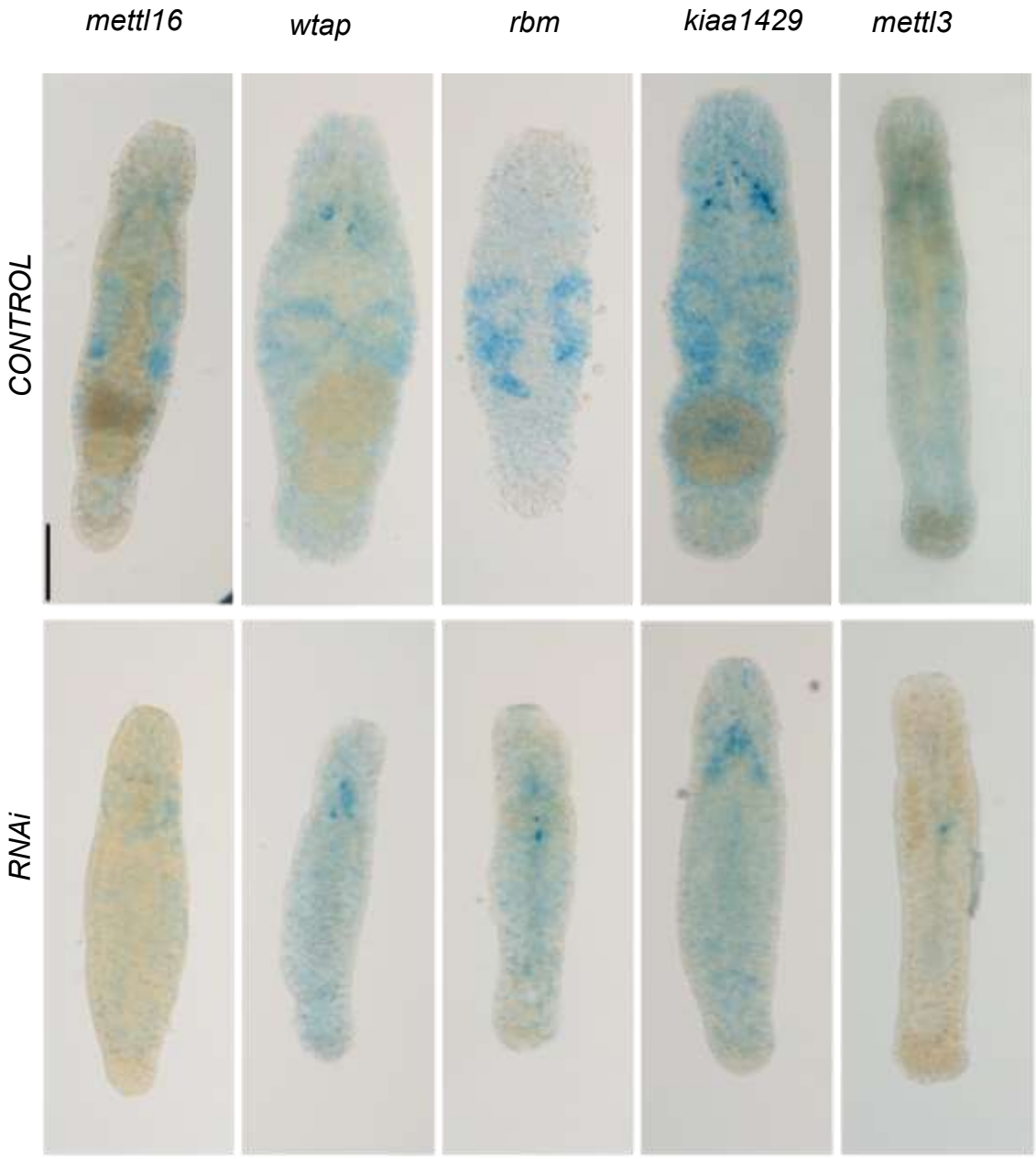
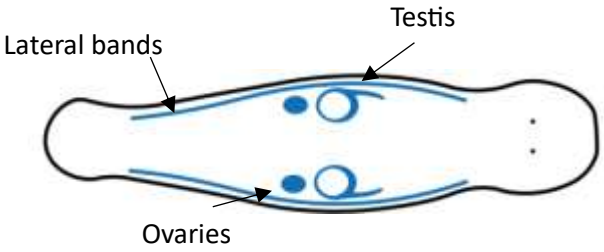


Fig 4.1.4: m6A Writer's expression pattern on homeostasis worms
Homeostasis worms developed with Vector Blue staining, after treatment with RNAi against the negative control (top) and the gene of interest (bottom).
Scale bar: 100 μm
In the cartoon are shown the regions where the genes expression is supposed to be.



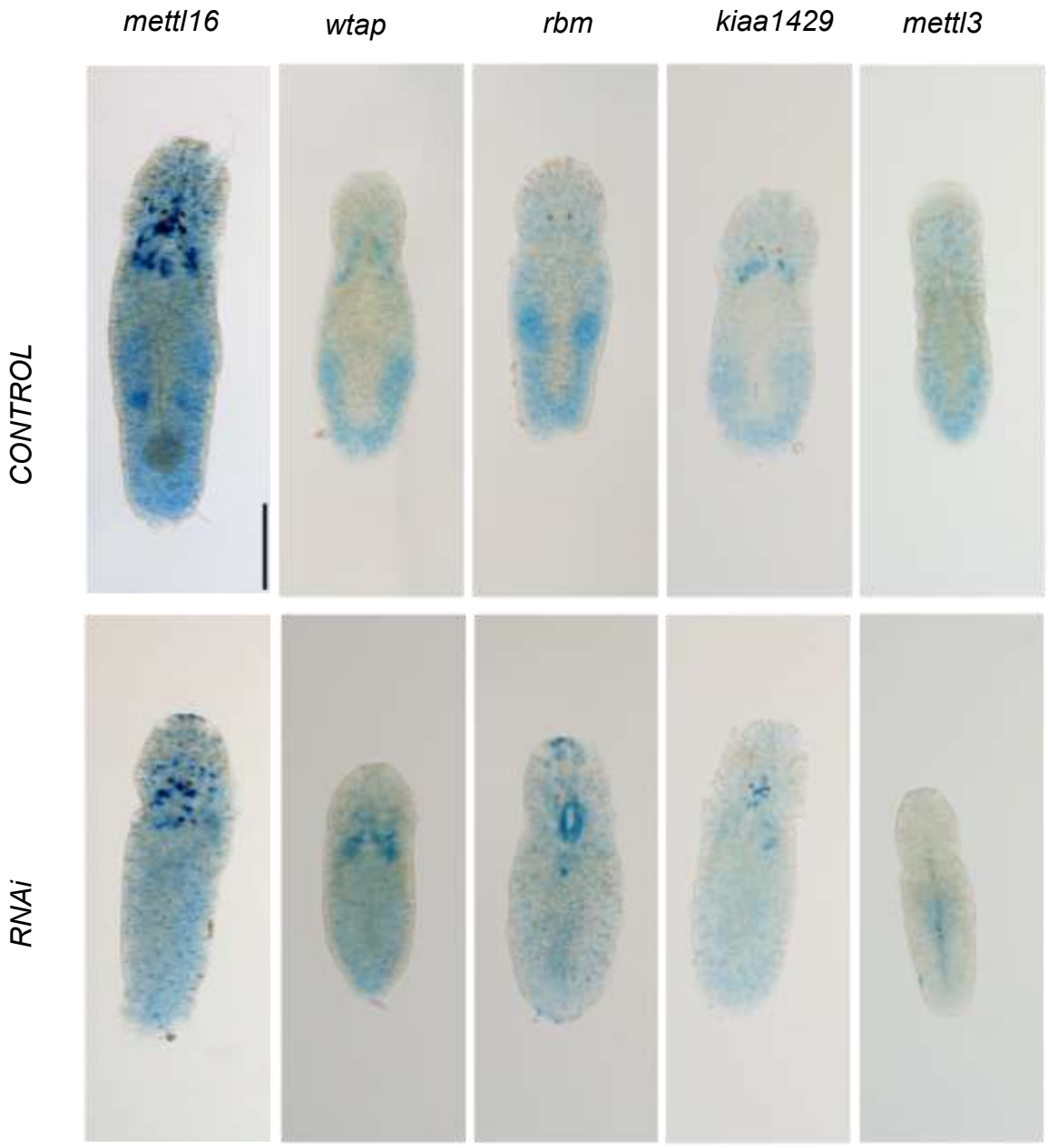
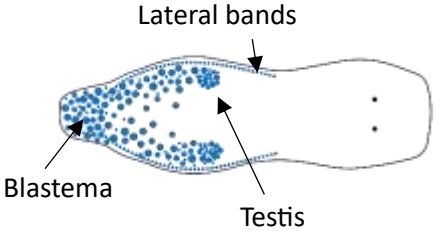


Fig 4.1.5: m6A Writer's expression pattern on amputated worms
Amputated worms developed with Vector Blue staining, after treatment with RNAi against the negative control (top) and the gene of interest (bottom).
Scale bar: 100 μm
In the cartoon are shown the regions where the genes expression is supposed to be.



Genes' expression patterns were confirmed to be enriched mainly in the gonad's region and less in the lateral bands in homeostasis worms. A ubiquitous signal can be observed through all the length of worms, that may be due to a small expression of the genes in differentiated cells or a nonspecific signal from the ISH procedure. This can be observed in homeostasis and amputated worms. Moreover, underneath the mouth are often present some dots of signal that could be related to the presence of salivary glands producing sticky mucus that could retain some probes/dsRNA.

All amputated worms displayed a strong signal in the blastema region, and in almost all cases we can see also some signal from a small portion of gonads that was not cut during amputation, recognizable as a small round area between the head and the blastema.

After 1 week of knockdown the signal from the gonads and the blastema was significantly diminished, but the background signal seems to be persistent in all worms, even though it is less intense in *Mlig-mettl16* homeostasis and *Mlig-mettl3* homeostasis and amputated worms. In addition, I've noted an unusual signal present in the middle of the worms, in the centre of the gut region, only in individuals treated with dsRNA against the gene of interest, which related probes were then added during the ISH procedure.

4.2: Gene knockdown

4.2.1: Knockdown of m6a writer's genes do not affect significantly embryonic development

Considering the involvement of m6A in the regulation of neoblast proliferation and differentiation, we wanted to see what would happen if we did a knockdown of the writers' genes during a phase in which these events are crucial: the embryonic development. The hypothesis behind this experiment was that, if indeed these genes are fundamental and expressed during embryonic development, the knockdown would result in an undeveloped embryo.

RNA interference on *M. lignano* eggs has never been tried before, so there is no official protocol to follow. We tried this experiment as a proof of concept by microinjecting the eggs, following the established microinjection protocol by (Wudarski et al. 2017), explained in section 3.3.7. We used the same stock aliquots created for the soaking techniques, each containing ≈200ng/μL of dsRNA. I added ≈1,5 μL of dsRNA solution in the needle, but considering that the machine injects femtoliters, it is hard to quantify how much dsRNA was injected in every single egg. For each gene, I considered a positive and negative control. The negative control was represented by a dsRNA designed against the *C. elegans* gene *heh1*, which is not present in *M. lignano*, while I used a dsRNA against *ddx39* as a positive control, a gene codifying a transcription factor that has been proven fatal also in adults. (Wudarski et al. 2019)

After injecting the eggs at day 0, I monitored the embryonic development until the eggs hatched, which was mainly between day 3 and 5. After the worms were born, I added diatoms to feed them and transferred them in a new well with fresh ASW and food once a week, for around 2 weeks, until the hatchlings became adults.

No statistically significant results were observed during the embryonic development; if a peak of 25% undeveloped eggs is observed in the *Mlig-kiaa* injected eggs, this did not result significant compared to the negative control and more interestingly, *Mlig-wtap* and *Mlig-rbm* presented a lower percentage of undeveloped eggs than the negative control. Indeed, a small percentage of non-developed eggs was always present also in the negative control, meaning that the injection procedure per se damages the eggs.

Moreover, no particular phenotypes were noted after the hatch of the eggs, all worms were able to grow at a normal rate and did not present morphological abnormalities.

Eggs were identified as "not developed" when they looked like the positive control in Fig.4.2.1 and didn't develop further the following weeks.

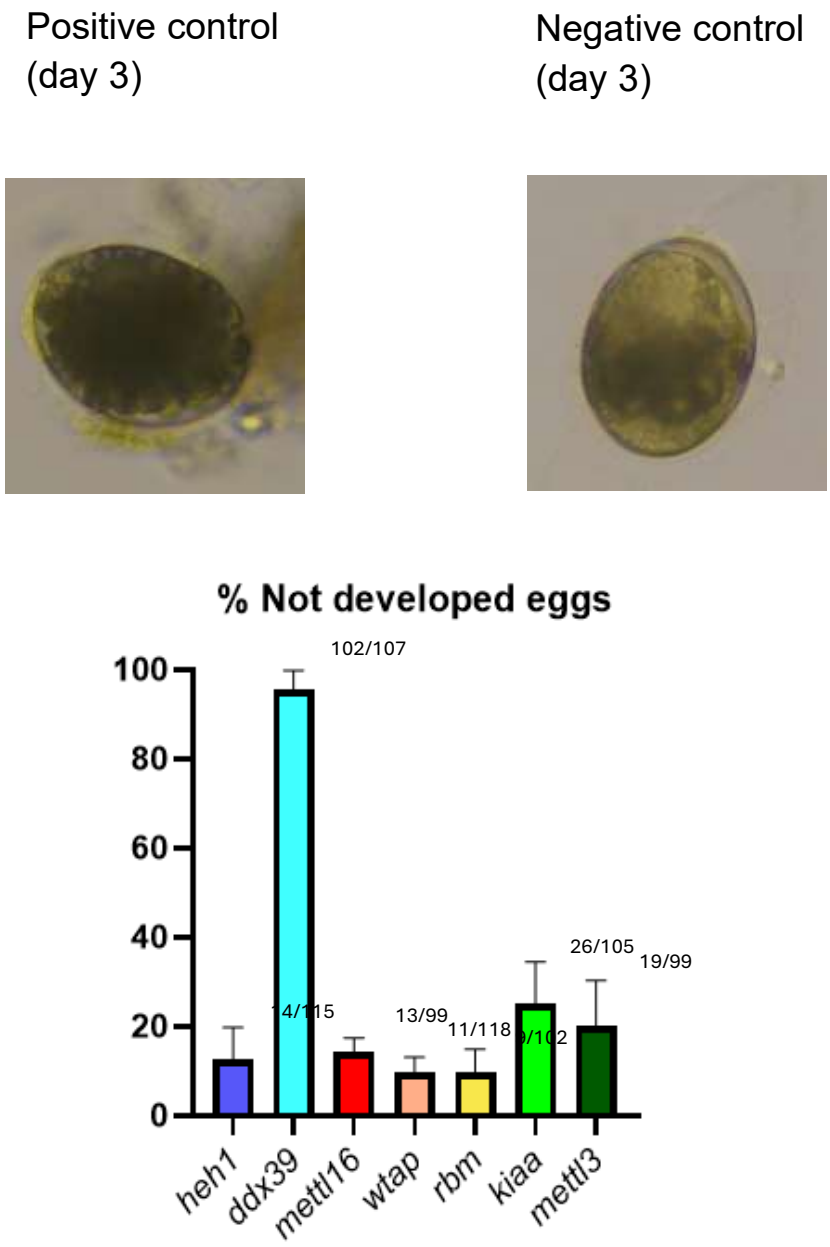


Fig. 4.2.1: Embryonic development – positive and negative controls at day 3

(A) Eggs injected with *ddx39* dsRNA could not complete embryonic development.

(B) Fully developed worm inside the egg after injection of *heh1* dsRNA. It can be recognized by the smooth edges and, depending on their orientation inside the egg, eyes can be observed, together with some movements.
Eggs were kept in the incubator at 25°C.

(C) Graphic representing the percentage of not developed eggs for each gene and specifying the number of not developed eggs on the total amount, for each gene. I performed a statistical analysis to assess if I had significant results. Since not all groups of data were normally distributed, I applied the Kruskal-Wallis test for non-parametric distributions and compared the means between each group and the negative control with Dunn's test. No comparison was statistically significant (supplementary materials Fig. 2).

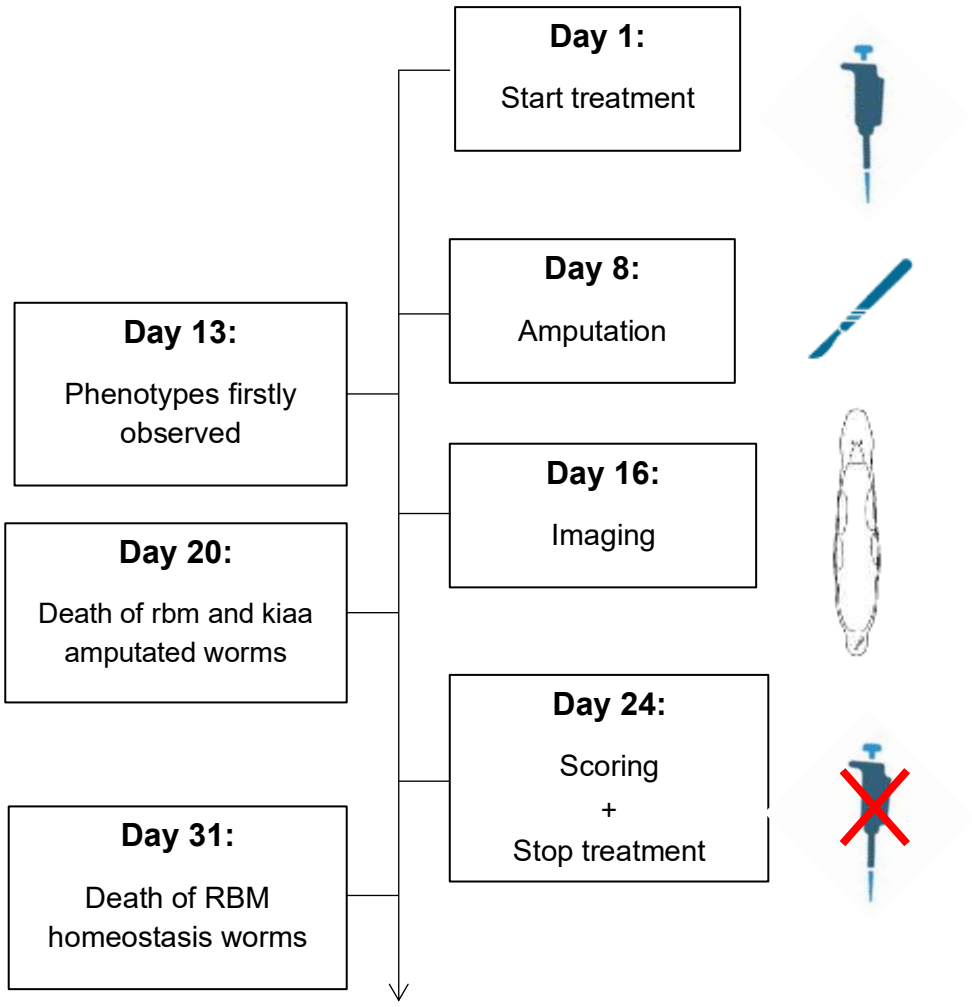
4.2.2: Knockdown of writer's genes affects significantly neoblast homeostasis and regeneration in *M. lignano* adults

I performed an RNAi experiment trough soaking following the appropriated protocol, using worms from the NL45 transgenic line. The experiment specifically targeted the genes involved in the deposition of m6A, respectively *Mlig-mettl3, Mlig-mettl14, Mlig-mettl16, Mlig-rbm, Mlig-kiaa1429, Mlig-hakai* and *Mlig-wtap.* The stock cultures containing the plasmid codifying for the dsRNAs were the same used by the previous student, with the exception of mettl3 that was newly created. As negative control I treated the worms with dsRNA designed for the *C.elegans heh1* gene. 35-40 worms per well were initially starved for 24h-48h, then treatment required soaking with the dsRNA solution twice a week for 24h and feeding the rest of the time, as better explained in the methods section (section….) Considering the first day of soaking as day 1, at day 8 half of the worms for each well was cut in the region between the gonads and the pharynx, to induce regeneration and study the effect of knockdown during this process.

I documented different phenotypes between the negative control and other genes, respectively for worms treated for *Mlig-rbm*, *Mlig-kiaa1429*, *Mlig-mettl16*, *Mlig-wtap*, related to their morphological aspect and fluorescence characteristics. The most evident phenotype in homeostasis worms was a change in the fluorescence, indeed I could observe in all of these worms a depletion of the signal, starting from the middle region, so where the more differentiated cells are, and gradually undergoing also on the sides. There was some variability between genes regarding the amount of signal depleted, and the number of worms that presented it, as it shows in Table 1. The amputated animals displayed hindered or slow regeneration and presented a lower intensity fluorescence signal, in some cases almost totally depleted, and wrinkled edges. *Mlig-rbm* and *Mlig-kiaa1429* amputated worms did not manage to regenerate and died within day 20. (Results fig. 4.2.3-7) After 3 weeks, I stopped the treatment and apart for *Mlig-rbm* homeostasis worms which died a week later, all the other animals managed to regenerate and re-establish a normal signal pattern and phenotype.

Interestingly, I did not observe a similar phenotype after knockdown of *Mlig-mettl3*. This triggered our curiosity more than the other genes, since it is the main responsible for the methylation. This is why we also decided to design a new dsRNA sequence for mettl3 which was longer and more specific, but still nothing changed. I also continued the treatment with mettl3 for two more weeks, to see if it required more time to show a phenotype, but no changes were registered.

Fig. 4.2.2: Timeline of the experiment



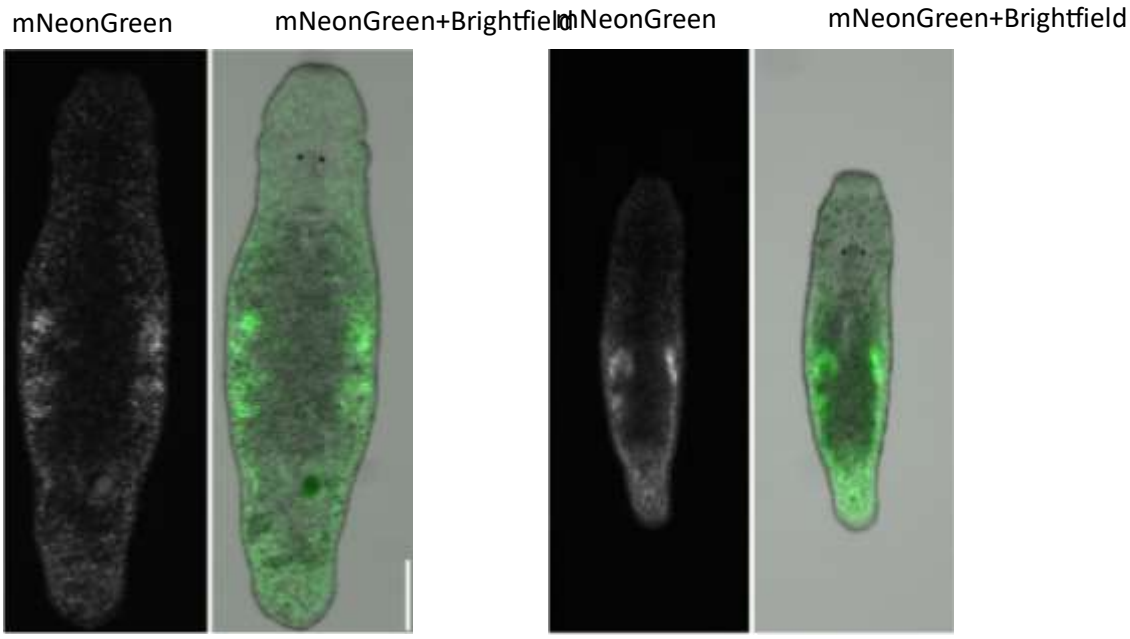
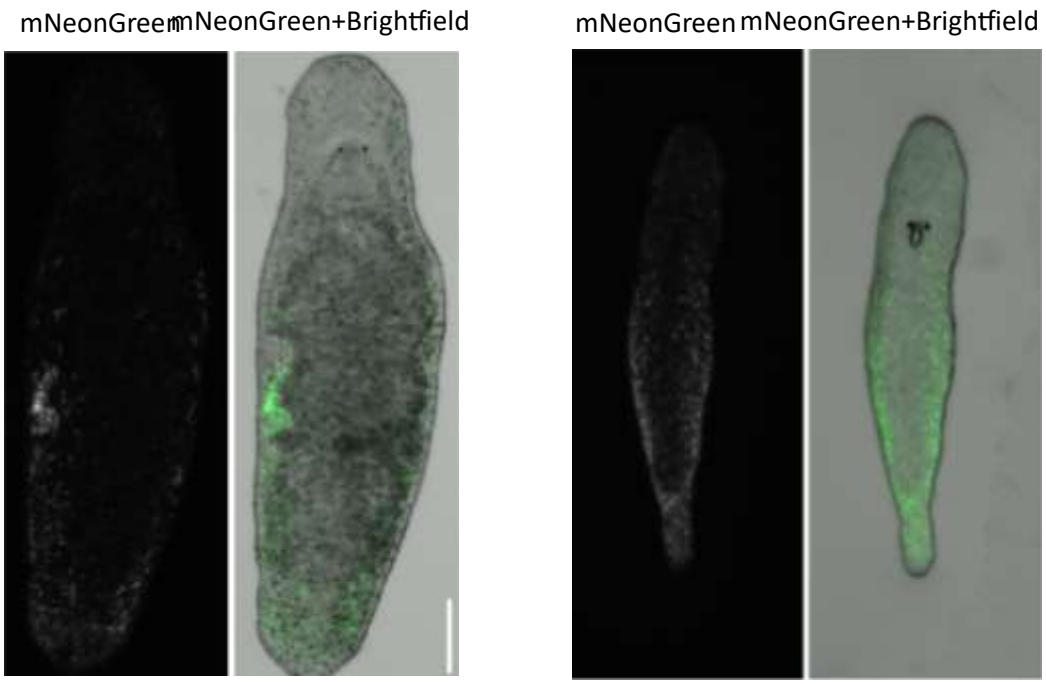


Fig 4.2.3: *heh1* negative control after 16 days of RNAi knockdown on NL45 worms

Homeostasis (A) and amputated (B) worms after treatment at day 16. After just one week the amputated worms presented a hint of gonads growth and a normal fluorescence signal pattern, showing physiological growing tail.

Scale bar: 100μm



(A) (B)

Fig 4.2.4: *Mlig-mettl16* after 16 days of RNAi knockdown on NL45 worms
Homeostasis (A) and amputated (B) worms after treatment at day 16.
(A)Homeostasis worms presented an important lack of signal mainly in the middle but weakly present also on the sides and in many cases the signal from the gonads was almost depleted.
(B) After one week the amputated worms presented a slightly lower fluorescence signal with a normal pattern, but no gonads.
Scale bar: 100 μm

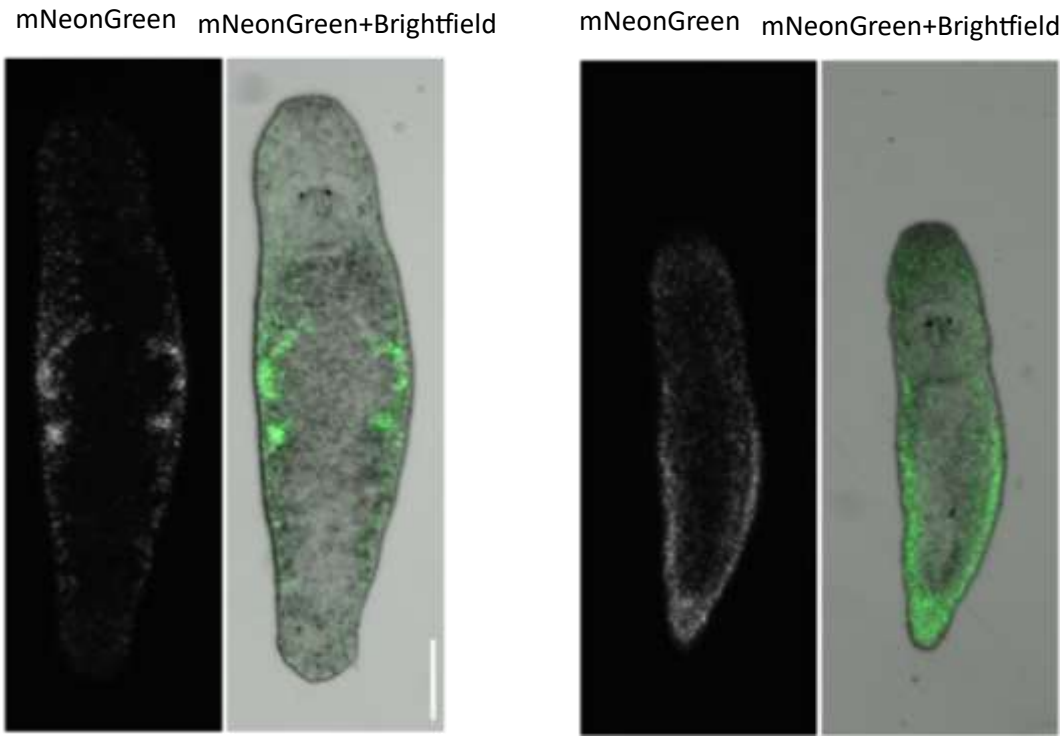


Fig 4.2.5: *Mlig-wtap* after 16 days of RNAi knockdown on NL45 worms
Homeostasis (A) and amputated (B) worms after treatment at day 16.
(A)Homeostasis worms presented an important lack of signal mainly in the middle but weakly present also on the sides and, in many cases, the signal from the gonads was almost depleted.
(B) After one week from the amputation, cut worms presented a slightly lower fluorescence signal with a normal pattern, but no gonads.
Scale bar: 100μm

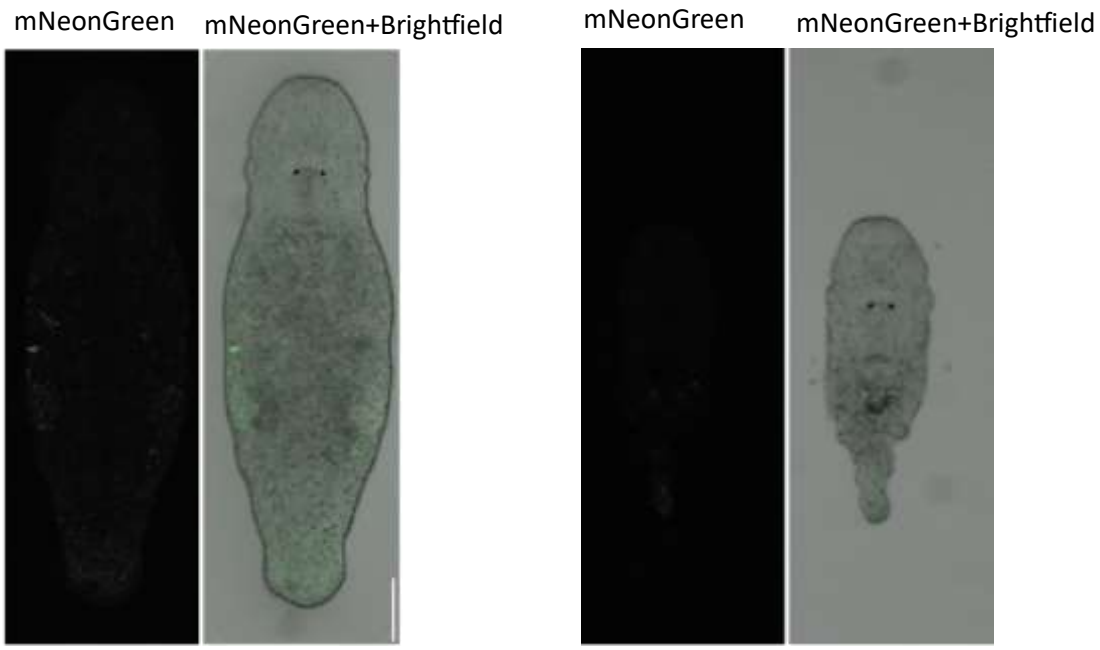


Fig 4.2.6: *Mlig-rbm* after 16 days of RNAi knockdown on NL45 worms
Homeostasis (A) and amputated (B) worms after treatment at day 16.
(A)Homeostasis worms presented a total depletion of the fluorescence signal. Indeed, a week after terminating the experiment they all died.
(B) After one week from the cut, the amputated worms presented almost no fluorescence and morphological differences from the control, showing wrinkled edges.
Scale bar: $100\mu m$

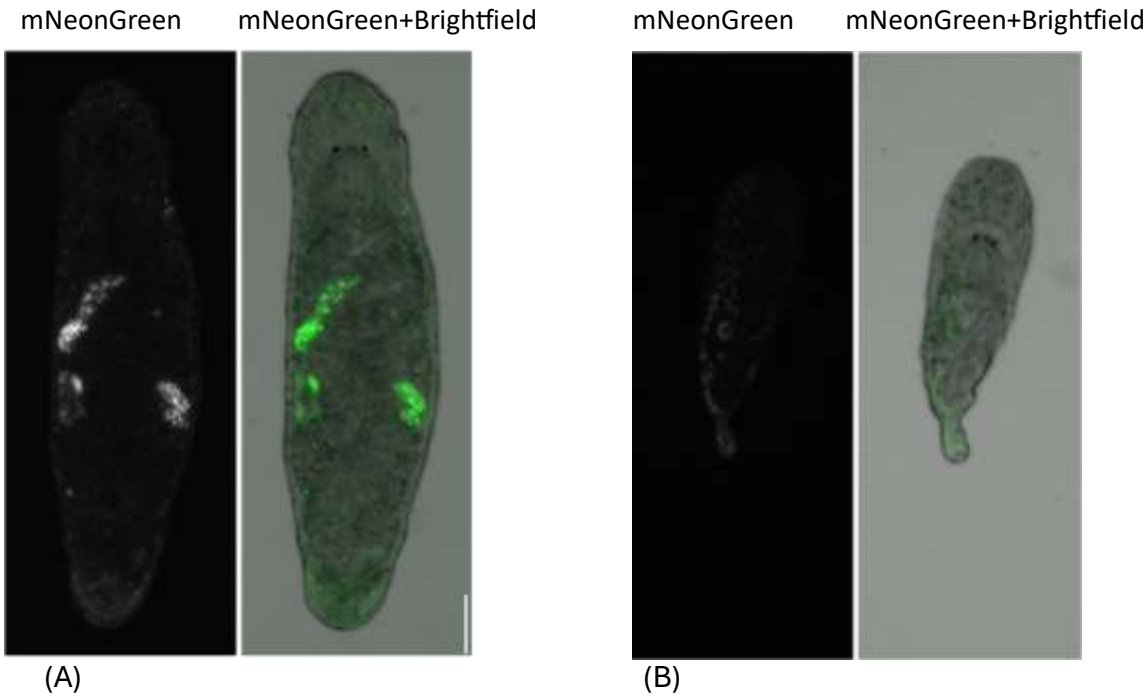


Fig 4.2.6: *Mlig-kiaa1429* after 16 days of RNAi knockdown on NL45 worms
Homeostasis (A) and amputated (B) worms after treatment at day 16.
(A)Homeostasis worms presented a nearly total depletion of the fluorescence signal.
(B) After one week from the amputation, the cut worms presented almost no fluorescence and morphological differences from the control, showing wrinkled edges.
Scale bar: 100μm

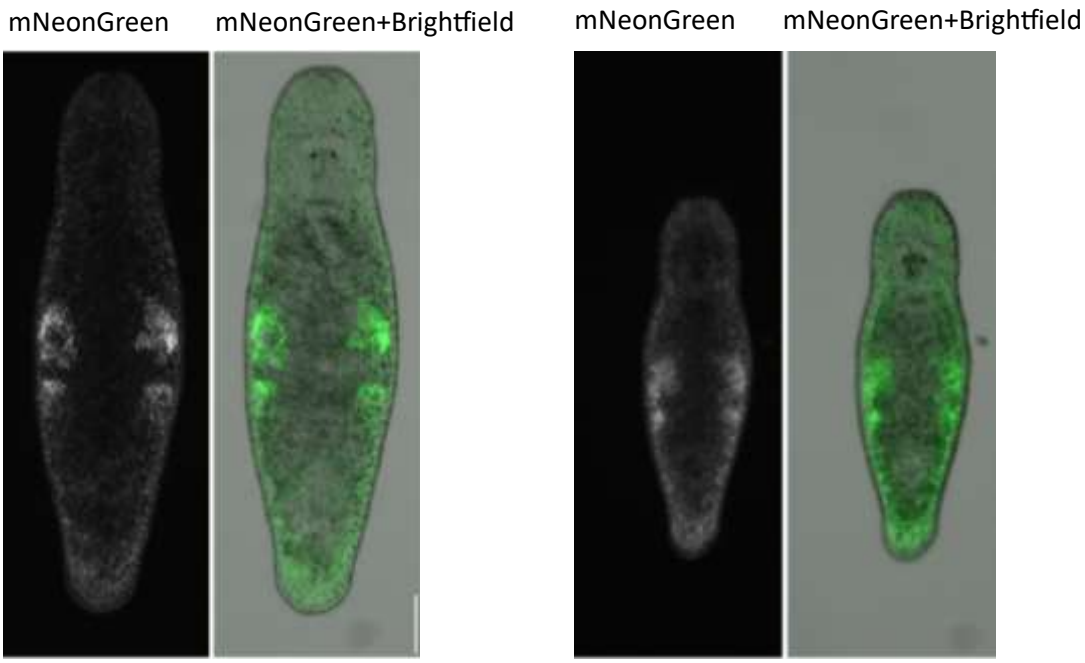


Fig 4.2.7: *Mlig-mettl3* after 16 days of RNAi knockdown on NL45 worms
Homeostasis (A) and amputated (B) worms after treatment at day 16.
(A)Homeostasis worms did not present any difference from the negative control.
(B) After one week from the amputation, the cut worms were normally regenerating and showed a standard fluorescence pattern.
Scale bar: 5100μm

Table 1: % Worms that displayed phenotypes

	Cut	Homeostasis
Neg. control (*heh1*)	0%	0%
mettl16	73%	78%
wtap	64%	60%
rbm	100% dead	100% dead
kiaa	85% dead	76%
mettl3	0%	0%

Four genes out of seven displayed a particular phenotype after RNAi knockdown. *Mlig-mettl16, Mlig-rbm, Mlig-kiaa1429* and *Mlig-mettl16* presented all the same signal-depletion pattern with some variability regarding the amount of worms which presented it and the degree of the loss of signal. The ratio of worms portraying the phenotype goes between 60% and 100% of individuals, with *Mlig-mettl16* and *Mlig-wtap* presenting the less sever abnormalities and *Mlig-rbm* and *Mlig-kiaa1429* the most penetrant ones.

Mlig-mettl16 and *Mlig-wtap* treated worms indeed, presented a slower regeneration in amputated worms that still managed to fully grow, and all homeostasis animals were able to recover a normal fluorescence pattern without encountering morphological modifications after stopping the treatment.

In all cases, the signal loss started from the centre of the worms, continuing to the sides and in the worst cases involving also the gonads, which happened mainly in *Mlig-rbm* worms, but in minor numbers also in the other cases. I could observe an almost total depletion of the fluorescence only in the *Mlig-rbm* treated worms and in *Mlig-kiaa* amputated animals, which indeed could not survive.

Morphological manifestations, as wrinkles on the sides, appeared a few days before the individual's death, probably as a sign of the last stage of cellular failure.

As it can be observed, no abnormal fluorescence signal or morphological appearance were present in the *Mlig-mettl3* treated worms, even by continuing the treatment for a longer time, and similarly, no phenotypes were registered for *Mlig-mettl14* and *Mlig-hakai.*

4.2.3 RNA interference on hatchlings impacts normal development

I finally investigated which effect RNA interference has on the writer's genes on hatchlings. In order to do this, I applied the same protocol used for adults and regenerated worms, using the same NL45 transgenic line.

I harvested 30-40 eggs for each gene, in a timespan of 3 days since I didn't have a culture big enough to get all the eggs in just one day. So, by the time I started the treatment the worms were about 2-4 days old. I used *heh1* as negative control and *Mlig-ddx39* as positive control. If we consider day 1 as the first day of treatment, at day 9 I started observing worms treated with *Mlig-ddx39 s*hrinking and presenting wrinkle edges, which died in 1-2 days, when they were about 11-13 days old (Fig 4.2.12). Moreover, I documented a slower development growth, even in the negative control group. Indeed, if usually it takes around 2 weeks to became adults after hatching, control worms took almost 3 weeks to fully grow. This was probably due to a technical problem related to my inexperience in feeding so many hatchlings and handling the diatoms cultures so that they don't cause toxicity. By day 22, I could observe the presence of 3 phenotypes: worms with a normal fluorescence signal and full-grown gonads (+signal/+gonads), worms with a normal signal but no gonads (+signal/-gonads) and worms with an almost depleted signal and no gonads (-signal/-gonads), the two latter both with a smaller body. Surprisingly, after stopping the treatment, I didn't register any worm dying, so they were all able to recover. The ratio of phenotypes differed between genes, as it is shown in Fig. 4.2.9-11, where, on top of the columns, are specified the number of worms with phenotypes on total worms.

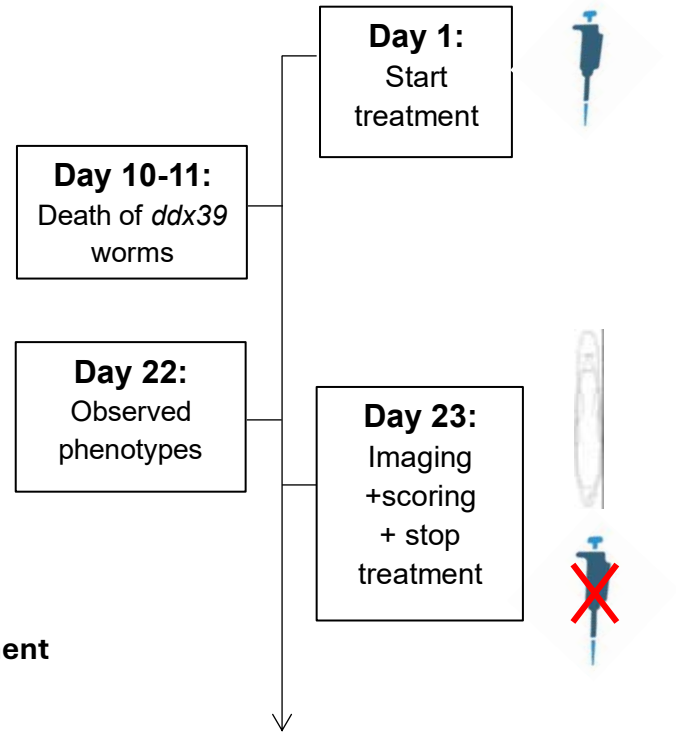


Fig 4.2.8: Timeline of the experiment

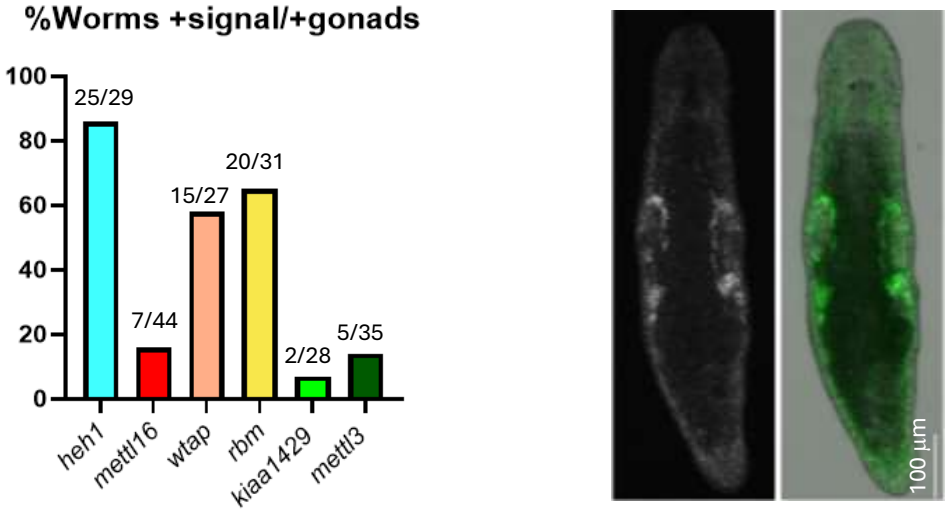


Fig.4.2.9: Worms +signal/+gonads

Adult size worms with regular fluorescence pattern and fully grown gonads.

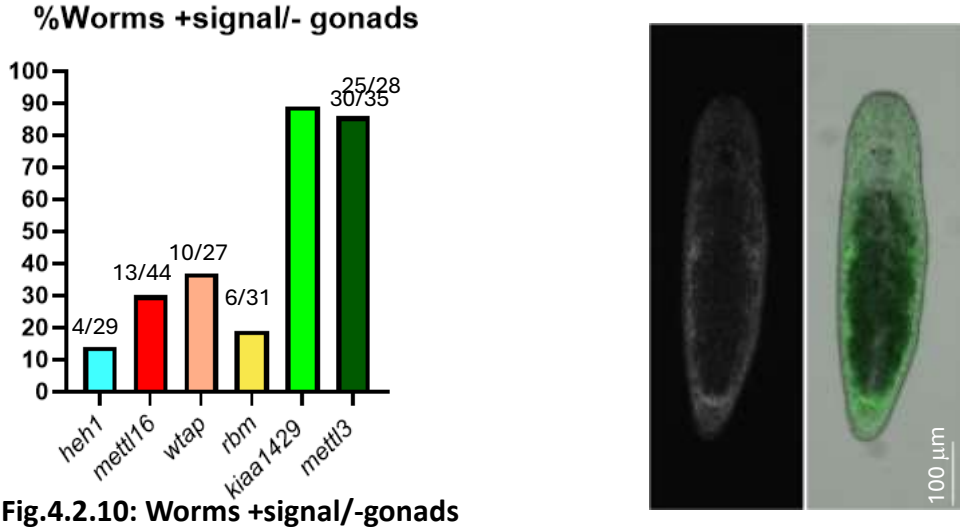


Fig.4.2.10: Worms +signal/-gonads

Short worms with normal fluorescence pattern but no gonads.

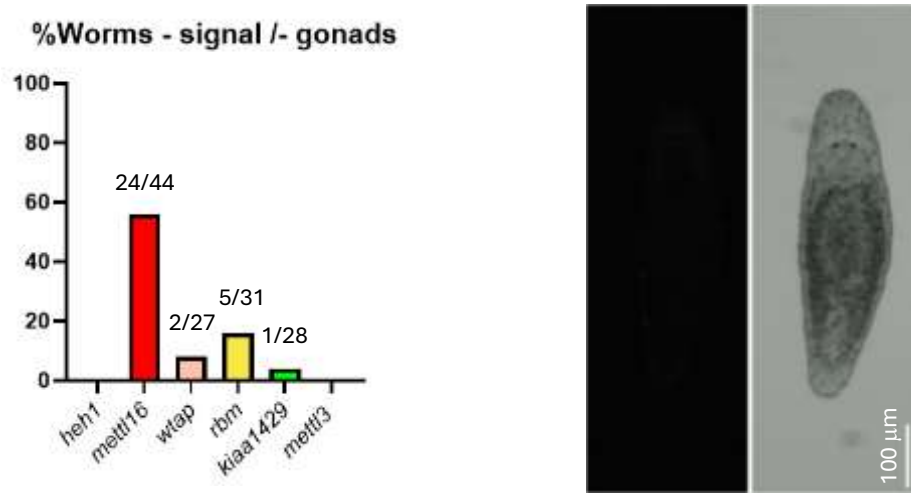


Fig.4.2.11: Worms -signal/-gonads

Short worms with an almost completely depleted fluorescence signal and no gonads.

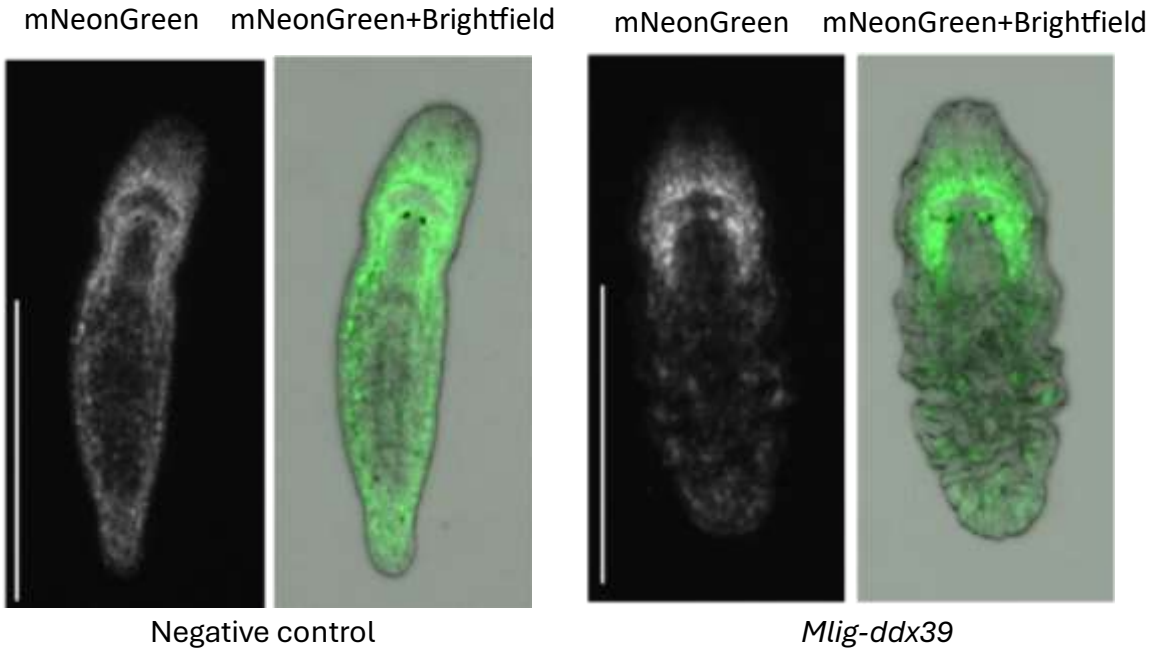


Fig. 4.2.12: Negative control and *ddx39* hatchlings at day 9
ddx39 treated worms displayed morphological abnormalities with a shrunk body and wrinkled edges and a different fluorescence signal from the control.
Scale bar: 100 μm

The two main phenotypes were the +signal/+gonads and +signal/-gonads, respectively represented by 74 and 88 worms out of 194, which are present with different percentages in each gene, even the negative control.
The +signal/+gonads worms present a fluorescent signal that would be expected in adult worms of the NL45 transgenic line, with a high intensity of fluorescence on the lateral bands and on the gonads, decreasing by going in the middle.
+signal/-gonads individuals, instead, are missing the fluorescence from the gonads, in a few cases just a small hint of signal is present near the testis location like in Fig. 4.2.10, and moreover present a smaller body compared to the first phenotype.
-signal/-gonads worms were almost completely depleted of the fluorescence signal and presented a significantly reduce body. The latter were only 32 in total, with a peak presence in *Mlig-mettl16,* and interestingly they were not present in the negative control and *Mlig-mettl3* treated animals.
Normal growing hatchlings, as represented by the negative control in Fig.4.2.12, are characterized by a really high intensity signal, mostly in the head region, due to the enhanced cell proliferation rate, which is present also in *ddx39* treated worms where, instead, the signal from the rest of the body is much lower.

Chapter 5: Discussion

5.1: RNA in situ hybridization shows the expression patterns of m6A writers' genes and validates RNAi efficacy

RNA in situ hybridization revealed m6A writers' expression pattern. The studied genes appeared in the expected regions (neoblasts populated areas), even though the signal from somatic neoblast was really faint in homeostasis worms (lateral bands), but much more intense in amputated individuals (blastema), so where neoblast proliferation is much more active.

A ubiquitous signal seems to be present in homeostasis, amputated, treated and non-treated animals. If it is possible that a lower expression could be present in a few differentiated cellular clusters, it is also likely that the worms were not correctly washed after the hybridization procedures, or that antibody/probes concentrations were excessive, hence, leading to non-specific bindings and creating a homogeneous non-specific signal. This can also happen if the final development lasts too long, and the low concentrated areas may become visible.

RNAi efficacy was observed after one week of treatment and seemed to be working properly. Indeed, signals from gonads and blastema are greatly decreased, even if the general signal is not totally depleted. This could be due to a time matter and a longer treatment period may be necessary to reach a completely lack of signal or be still related to the non-specific issue already mentioned. Furthermore, in many cases I observed the presence of a linear pattern in the middle of the worms' body. Specifically, it seems to be localized in the centre of the gut. This signal only appeared in treated animals that were destined to visualize the knockdown, so subjected to the binding of the primary probe that recognized the same molecule of the related dsRNA. The most feasible hypothesis is then that a small amount of dsRNAs gets stuck in the diatoms or mucus in the worm's gut, and then is recognized by the probes.

Thanks to this ISH experiment, I was able characterize the expression localization of the genes of interest, and to confirm the efficacy of the novel protocol for knockdown by RNA interference, validating the results obtained from the RNAi treatment.

5.2 Knockdown of m6A writers interferes with the normal regulation of neoblasts proliferation and differentiation and hinders regeneration in adult worms

Knockdown of the writer's genes confirmed the previous results obtained, except for a few differences.

It was firstly noted by the previous student an upsurge of proliferating cells followed by their decline, by observing the intensity of the fluorescence signal. From this data a model was hypothesized which implied the presence of two separate stages that follows progenitor inability to differentiate: the first phase would be characterized by an increase of neoblast proliferation induced by a crosstalk between differentiated cells and progenitor, followed by the neoblasts death, so phase two. Since I did not observe the same phenomenon, I could not confirm the same speculated model.

Secondly, the first experiment was performed both with and old soaking protocol and electroporation, the latter implying treating the worms with a 60V square-wave pulse lasting 20 ms for 4 times in 15 days, as a method to deliver the dsRNA molecules. The old soaking protocol, instead, included soaking the worms every day in wells filled with ASW and diatoms, adding a dsRNA solution with half of the concentration that I used but, as I already mentioned, diatoms obstacle the delivery of dsRNA.

Interestingly, the results that I obtained with the new soaking protocol were more similar to the ones observed previously from the electroporation experiment, even though I never detected the two-stage phenomenon aforementioned. It is possible that the signal upsurge observed in Dana's experiments may be attributed to the damaging effects of electroporation on all cell types, unlike soaking which primarily affects proliferating cells. This increased cellular damage likely triggers a compensatory response, necessitating accelerated neoblast differentiation. In contrast, soaking induces a gradual cell death, leading to a more balanced rate of neoblast differentiation.

The general lack of signal that I observed in the homeostasis worms can be interpreted as the following: since it started from the region where differentiated cells are, the first effect that seems to be hampered is differentiation, as cells following their normal cycle physiologically die, they are not substituted, hence the loss of signal; the second phase where also fluorescence from the neoblast region is lost, represents impossibility to proliferate and death of stem cells.

The same explanation could justify the slow regeneration or death in amputated worms.

The differences in the phenotypes between homeostasis and amputated worms treated for *Mlig-mettl16* and *Mlig-wtap* and the ones treated for *Mlig-rbm and Mlig-kiaa,* could be explained by the fact that the first genes are maybe not as crucial for methylation as much as the others, which resulted in death of almost all regenerating worms.

The variability between the number of worms presenting phenotypes and the between the amount time of the loss of signal, can be explained by the different efficacy of RNAi for each gene given by the possible presence of unknown off targets, as much as the diverse levels of expression of each of them.

At the end, the phenotypes registered in both mine and Dana's experiments were related to the same genes, which validates more the results, since the experiment was performed by two different people, years apart and by applying different protocols.

The missing phenotype from the *Mlig-mettl3* knockdown could be the reflection of a different m6A regulation from the one that has been deeply studied so far, so in vertebrates and specifically mammals, which, if we consider the evolutionary distance from *Macrostomorpha* and the beforementioned groups, is quite likely. As support of this hypothesis, no published studies in evolutionary closer clades like planaria, so far, has reported phenotypes related to the knockdown of *Mlig-mettl3,* so maybe it does not preserve the same importance.

In light of these circumstances, I think it would be useful to try an RNAi experiment where dsRNA against *Mlig-mettl3* and *Mlig-mett14* are combined in the same well, since maybe *Mlig-mettl14* presents a more active role in this organism than it does in other species. We also cannot exclude the presence of another methyltransferase that is yet to be discovered.

Differently, if we consider METTL3 to be indeed the most important protein for m6A deposition, another reason why RNAi against *Mlig-mettl3* didn't work could be the presence of a compensation mechanism. Regarding this, analysing the expression of all the writers related proteins, before and after the knockdown, could show how the whole complex responds to the knockdown of one of their components.

We cannot leave out also the technical aspect related to the fact that RNAi knockdowns never have a 100% efficacy. We know that *Mlig-mettl3* is not highly expressed as it can be visualized in the single cell data library and considering that RNA interference works better with high expressed genes, it is logical to think that the knockdown could not be strong enough to interfere with the normal METTL3 activity. Following these considerations, I suggest that performing knockouts could shed more light on the importance of this protein and to all the proteins studied.

As a final consideration to this experiment, I cannot yet declare that the phenotypes that I observed are related to a decrease of the levels of m6A, since I don't have a sequencing analysis to confirm that, even though it is what I am speculating, but I can for sure state that the knockdown of the proteins related to m6A deposition interferes with the normal regulation of neoblasts proliferation and differentiation, strongly hindering the regeneration process.

5.3 Knockdown of m6a writer's genes during embryological development opens new questions about their regulation

As anticipated, no established protocol has been developed for RNAi on *M.lignano* eggs, but since a protocol for injections is available, we decided to use it as delivery method for the dsRNAs.
The first problem related to the injection, is that there is no confirmation that the egg is correctly injected. One way to visually confirm that, is to see a small "cloud" inside the eggs while injecting, but this not always works. A good solution would be to use eggs from an NL45 culture, which present fluorescence already at the zygotic phase, and to inject dsRNA against mNeonGreen together with the dsRNA for the gene of interest, so that the correctly injected eggs would be recognized by a low intensity fluorescence signal. Unfortunately, for technical and timing issues I couldn't manage to apply this technique that could prove efficient for RNAi.

The results that I obtained didn't show any statistically significant difference, but some considerations must be taken into account.
First of all, we don't know if and how much the studied genes are expressed during this stage, their regulation could vary between the life history of the worms, hypothesis that the experiments with adults and hatchlings could also support. Secondly, the embryonic development is quite fast (3 days at 25°C), and the zygote usually retains proteins from the mother that could prevent the effect of knockdown even if it technically works and mRNAs are depleted.
Furthermore, we cannot exclude that the undeveloped eggs weren't the result of the knockdown, if it is true indeed that also in the negative control a small percentage of eggs did not develop (≈12%), each injection is an independent event and the variability given by the fragility of eggs, together with a possible error within the injection machine which pressure could be slightly different between events, and the expertise of the operator, are all factors that participate in the variability of the environmental conditions experienced by each egg.

Moreover, the data from the positive control gives us an important insight about the efficiency of RNAi and the actual rate of success of injections. Indeed, almost all eggs displayed impossibility to develop which could mean that also a great amount of all the other eggs were correctly injected.
With this premises, we can look at the results with a different light and consider that the lack of significant data is maybe not mainly given by a technical problem but could be the reflection of a biological matter that needs to be further investigate.
Finally, to deeply study this, I would suggest performing knockouts of the studied genes, as a more drastic way to understand the importance of each protein.

5.4 RNA interference on hatchlings impacts normal development

The RNA interference experiment performed on hatchlings is maybe the hardest to interpret. Indeed, I could conduct just one replica of it, and I encountered some troubles.
The only previous experiment of RNAi on hatchling was performed in 2008 by Pfister et al. where they investigated the expression of the *vasa* gene homolog in *M. lignano, macvasa,* marker gene for germinal cells, which after 6 days of knockout by RNAi resulted significantly decreased. Nevertheless, no previous RNAi was executed on hatchlings following the latest RNAi protocol, that's why the positive control represented the first really important aspect to observe since the death of the individuals treated with *Mlig-ddx39* showed the efficacy of RNAi at this stage of the worm's life.
The major issue that I went through was related to the feeding: to understand how much diatoms need to be sown is really important, since too many could overgrow and start producing domoic acids which is toxic to the worms, and a too little quantity would cause starvation resulting in changes in genes expression, interfering with the validity of the experiment.
After seeing that in 15 days all the worms, also the negative control, were still juveniles while they were supposed to be adults, made me realize that they probably had not been fed enough. By adapting the amount of diatoms sown, from that day all the worms managed to grow much faster, with the control individuals reaching adulthood in one week.

The results must be interpreted considering the different ratio of phenotypes per gene. If the +signal/-gonads phenotype was the most represented, it also could have been the result of the starvation, as its presence also in the control group could prove. The -signal/-gonads phenotype, instead, has never been observed before associated to starvation, and it is not present in the control group and the

Mlig-mettl3 treated animals, so it is the most likely variation given by the knockdown.
In light of these circumstances, I hypothesize that starvation interfered with the normal genes' expression, indeed, since neoblasts proliferation was reduced due to lack of food, it would be logical to think that the genes involved in this process were downregulated. Considering that RNAi works better with higher expressed genes, the effect of knockdown was then limited. The worms that actually displayed a penetrant phenotype were maybe the ones who managed to eat a greater amount of food than the others or presented an unknown condition that exposed them more to knockdown.
Nevertheless, given the observed phenotypes, it would seem that the studied genes have an important role during development, regarding neoblast homeostasis and ability to proliferate.
The fact that no worms died is indeed an interesting event, considering that the same phenotype in adults resulted in death. My hypothesis is that neoblast proliferation in this stage of life is higher, and so the amount of neoblast needed to survive may be smaller than in adults.

To better investigate this, I suggest the following approaches: more replicas of the experiment are for sure needed to validate or not the results that I obtained, and further, an ISH analysis on hatchling and juveniles would light more information regarding the expressions of the studied genes and the efficacy of RNAi on them, together with a knock-out experiment.

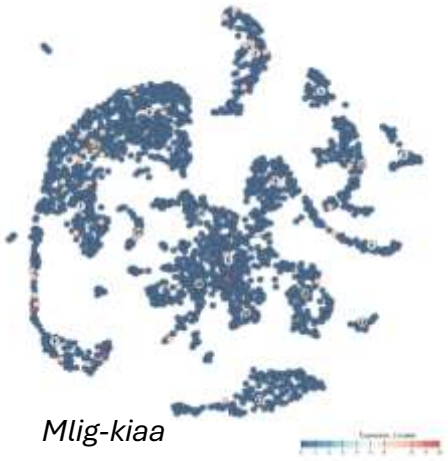
Chapter 6: Conclusions

This project aim was to better understand the role of m6A methylation in the stem cell population of a novel model organism.
M. lignano and its expanding toolbox allowed us to investigate the dynamics of this epitranscriptomic modification in different conditions.
Thanks to the NL45 transgenic line, we were able to visually observe how the RNAi knockdown of the m6A writers' genes affects the activity of neoblast in the worms' physiology during development, regeneration and homeostasis. Microinjections allowed us to explore this process also during the embryonic development, while the new ISH method gave us important insights in establishing the expression pattern of the genes of interest.
Overall, these tools permitted us to confirm previous obtained data, validate newly developed methods and further explore the importance of m6A in a model organism where this modification has never been studied before.

I believe that the performed experiments collectively contributed into a more profound understanding of m6A regulation, raising questions that will help pursuing the research on this topic.

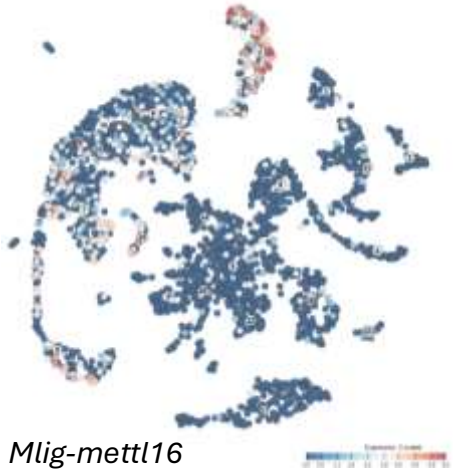
Supplementary materials

Fig. 1: Single cell atlas for the m6A writer's genes

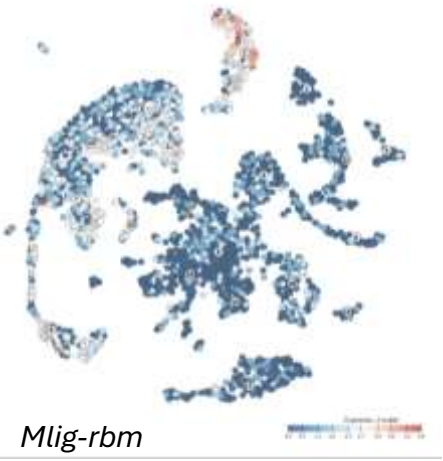


Mlig-kiaa

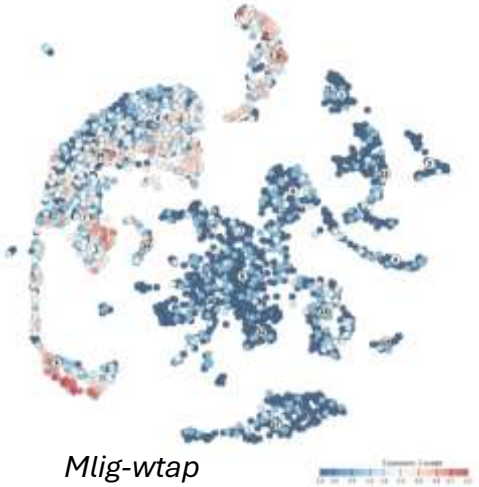
Mlig-mettl3



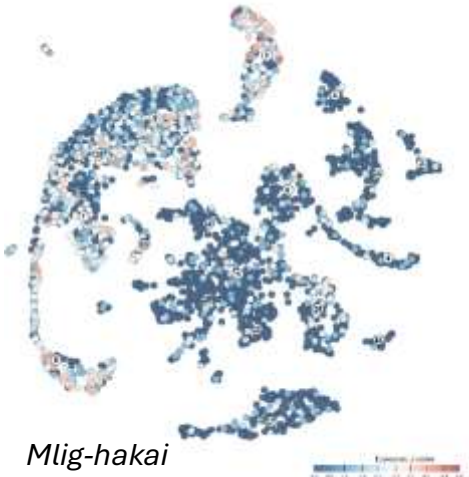
Mlig-mettl16



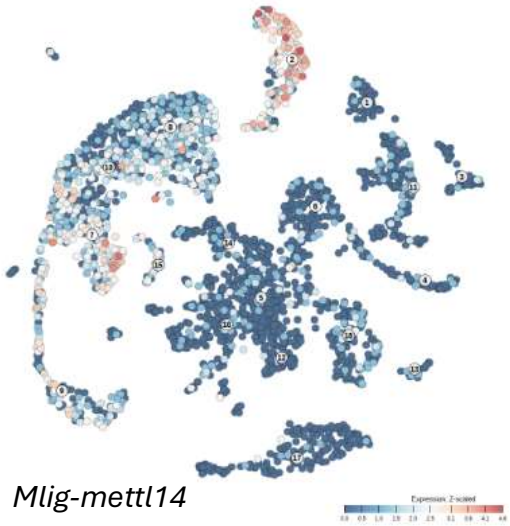
Mlig-rbm



Mlig-wtap



Mlig-hakai



Mlig-mettl14

Fig. 2: Statistical analysis: comparisons between injections

Dunn's multiple comparisons test	**Adjusted P Value**
heh1 vs. mettl16	>0,9999
heh1 vs. wtap	>0,9999
heh1 vs. rbm	>0,9999
heh1 vs. kiaa	0,5015
heh1 vs. mettl3	>0,9999
Alpha	0,05

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