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ROLE OF SIGNALING PATHWAYS IN MAINTENANCE AND PERIODIC ACTIVITY OF POSTERIOR STEM CELLS IN THE ANNELID *PLATYNEREIS DUMERILII*

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Role of signaling pathways in maintenance and periodic activity of posterior stem cells in the annelid *Platynereis dumerilii*

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Summary in Italian

Introduzione

La segmentazione dell'asse corporeo è una sorprendente proprietà morfologica che accomuna alcuni gruppi di animali. In particolare, questa proprietà è riscontrabile negli anellidi, negli artropodi e nei vertebrati. L'origine della presenza di un asse anteroposteriore segmentato fra gli animali a simmetria bilaterale è una domanda biologica emersa precocemente nella comunità scientifica. In particolare, ci si è chiesto se l'antenato comune a tutti gli animali a simmetria bilaterale, definito "Urbilateria", fosse segmentato o meno.

L'ipotesi tradizionale prevede uno stato ancestrale non segmentato e diversi casi di convergenza evolutiva che hanno portato gruppi distanti a presentare una morfologia simile. Questa ipotesi è avvalorata dalla mancanza di conservazione dei processi biologici che portano alla comparsa di un asse anteroposteriore segmentato. Tuttavia, alcuni dei phyla che includono specie segmentate sono stati ancora poco studiati. Infatti, la segmentazione tipica degli anellidi è morfologicamente simile a quella degli artropodi, presentando entrambi una segmentazione che coinvolge sia l'ectoderma sia il mesoderma. Negli artropodi risulta chiaro che l'ectoderma segmentato induce la segmentazione dei tessuti mesodermici e si sospetta che nell'anellide possa esistere un meccanismo simile. Al contrario la segmentazione dei vertebrati è dettata a livello mesodermico e si estende ad una parte dell'ectoderma.

Un modello tradizionale di filogenesi proposto da Lamarck, basato su caratteri prettamente morfologici come la segmentazione dell'ectoderma, prevede delle distanze evolutive ridotte fra gli artropodi e gli anellidi, al punto da poterli condensare in un unico gruppo, gli "Articulata". La ridotta divergenza fra i due gruppi, unita alle profonde differenze rispetto ai vertebrati, suggeriscono che la segmentazione sia emersa nell'arco della storia evolutiva due volte in maniera indipendente.

Tuttavia, uno studio del 1995 sul DNA ribosomale ha messo in luce una distanza molto ampia fra i due gruppi. Questo ha pertanto rivoluzionato la percezione della comunità scientifica riguardo alla segmentazione di "Urbilateria". Pur non escludendo il fatto che la segmentazione dell'asse anteroposteriore possa costituire un vantaggio evolutivo per qualche ragione, e quindi possa essere stato selezionato in diversi casi, se i meccanismi genetici e molecolari sono conservati è piuttosto improbabile che network così complessi

siano nati più volte in maniera indipendente. Questo suggerirebbe dunque uno stato ancestrale segmentato, che si è man mano diversificato fino a perdersi del tutto in alcuni phyla nel corso dell'evoluzione. Ad ogni modo, la tesi dell'"Urbilateria non segmentato" non è ancora stata definitivamente scartata in ragione dell'alta pleiotropia delle vie di segnale coinvolte in questi processi.

Studi su *Platynereis dumerilii*, un anellide segmentato che è possibile allevare in laboratorio, sono stati cruciali nell'individuare dei processi biologici altamente conservati fra le diverse specie segmentate. In particolare, il meccanismo di definizione della polarità dei segmenti (*segment polarity genes*) si è dimostrato conservato fra le specie segmentate.

Platynereis dumerilii è un modello eccellente per lo studio dei processi di segmentazione e per l'osservazione delle cellule staminali in una nicchia non perturbata. Infatti, alcuni studi mostrano che l'aggiunta dei segmenti, che avviene durante l'intero ciclo vitale dell'invertebrato, avviene interamente mediante due anelli concentrici di cellule, localizzati nella parte sub-terminale dell'anellide. Queste cellule sono definite 'teloblasti'; sono in grado di dare origine a tutti i tessuti dell'individuo eccetto la linea germinale, possono essere trasformate con strumenti di modifica genomica come Tol2 e CRISPR/Cas9 ed infine è possibile effettuare esperimenti di microscopia confocale o spinning disk per misurare il livello o la localizzazione di espressione di proteine marcate con proteine fluorescenti.

Materiali e metodi

Microiniezioni

Per condurre una parte cospicua degli esperimenti di questo lavoro, ho dovuto far pratica con la microiniezione di zigoti di *Platynereis dumerilii*. Un operatore particolarmente allenato è in grado di microiniettare circa 150 uova per sessione. Per questo motivo, dopo essermi allenato, ho condiviso il lavoro di microiniezione con Guillaume Balavoine.

Ottimizzazione di un reporter fluorescente del ciclo cellulare (FUCCI)

Il gruppo di ricerca di Guillaume Balavoine ha prodotto dei reporter fluorescenti del ciclo cellulare basati sull'ubiquitinazione di proteine (FUCCI). Le proteine dei costrutti FUCCI sono proteine caratteristiche delle differenti fasi del ciclo cellulare, fuse con proteine fluorescenti. La comparsa o la scomparsa del segnale fluorescente indica la fase del ciclo cellulare. Ci si aspetta che le mitosi nei teloblasti siano coordinate e che procedano dall'area

ventrale verso quella dorsale. La squadra di Balavoine aveva già testato le singole proteine di fusione; io sono stato coinvolto nel test di un costrutto tricistronico dove tre reporter di differenti fasi del ciclo cellulare sono assemblate insieme, separate da un *ribosome skip site* (fig. 9). Dopo aver trasformato degli embrioni mediante la tecnica di trasposizione Tol2, ho acquisito delle immagini al microscopio confocale (15 hpf). In questo modo ho convalidato il funzionamento del costrutto fluorescente ed ho contribuito a proporre alcune soluzioni per ottimizzarne la resa. Una volta che il costrutto sarà perfezionato e rifinito, si punterà ad ottenere degli individui marcati sui teloblasti, per verificare la correlazione fra l'inibizione della via di segnalazione Wnt e la progressione del ciclo cellulare nei teloblasti.

Studio della ciclicità trascrizionale di alcuni geni

A partire da quanto noto in altri modelli come *Mus musculus* e *Danio rerio*, si sospetta che una parte dei geni Hes ed il gene Caudal (talvolta indicato come Cad o Cdx) siano trascritti con ritmo oscillatorio. Il secondo obiettivo del progetto è quello di trovare un gene reporter dell'attività oscillatoria in *Platynereis*. Alcuni dei geni della grande famiglia dei geni Hes in Platynereis sono espressi nei teloblasti. Lo studio, tramite ibridazione in situ, della trascrizione differenziale di questi mRNA consente di determinare potenziali geni candidati ad essere dei reporter dell'attività oscillatoria. Una volta stabilito quali geni siano trascritti ciclicamente, si procederà col marcare in fluorescenza la corrispondente proteina.

Marcare il recettore Notch con EGFP

Il recettore transmembrana Notch è noto per essere una proteina rilevante nella sincronizzazione dell'orologio segmentale nei vertebrati. Dal momento che il recettore Notch è trascritto in tutte le cellule e le ibridazioni in situ non forniscono risultati soddisfacenti, si è pensato di marcare il recettore Notch con una proteina fluorescente. Si è deciso di marcare il dominio intracellulare di Notch, perché un'attivazione della via di segnale risulti in un cambio di localizzazione del segnale fluorescente (fig. 11). Infatti, il dominio intracellulare di Notch (NICD) è trasferito al nucleo quando la via è attiva. La marcatura del dominio intracellulare di Notch è stata effettuata utilizzando la tecnica CRISPR/Cas9 Knock-in, ancora non pienamente sviluppata in *Platynereis*.

Risultati

Ottimizzazione di un reporter fluorescente del ciclo cellulare (FUCCI)

Il costrutto tricistronico testato è un ottimo reporter del ciclo cellulare. Le proteine sono ben separate fra di loro ed è possibile distinguere le differenti fasi del ciclo cellulare. Tuttavia, due dei canali presentano ancora un'intensità di segnale particolarmente debole (Fig. 15). Per aumentare il segnale dei due canali interessati, ho proposto di cambiare l'ordine delle proteine nel costrutto o la natura dei *ribosome skip sites*.

Studio della ciclicità trascrizionale di alcuni geni

Fra tutti i geni candidati, due di questi sono risultati particolarmente adatti come reporter di attività oscillatoria nei teloblasti. Il gene caudal sembra presentare almeno tre cicli di espressione: nell'intestino, nel pigidio (parte terminale dell'anellide) e nei teloblasti (Figg. 17-18). La microscopia confocale aiuta a visualizzare in maniera particolarmente chiara le interruzioni di espressione nell'anello dei teloblasti (Fig. 20). Il gene Hes2 sembra essere un reporter di attività oscillatoria più semplice, presentando soltanto anelli incompleti nei teloblasti (Figg. 19-22).

Marcare il recettore Notch con EGFP

L'esperimento di *knock-in* ha prodotto dei risultati positivi, con alcune aree ben marcate (Fig. 28). Quindi, per la prima volta, è stato possibile inserire una proteina fluorescente nel genoma di *Platynereis* utilizzando CRISPR/Cas9. L'esperimento presenta tuttavia alcune criticità e deve essere sviluppato ulteriormente: per esempio, abbiamo provato che il DNA iniettato senza la proteina Cas9 non è espresso, ma non abbiamo genotipizzato gli embrioni fluorescenti per essere sicuri che la proteina fluorescente fosse espressa a partire da un contesto genomico corretto. Inoltre, nessuno degli embrioni ottenuti è destinato a svilupparsi correttamente. Per questo motivo, ho proposto di ridurre la concentrazione di Cas9 utilizzata per il knock-in, al fine di diminuire la tossicità delle particelle ribonucleoproteiche. Se la concentrazione delle particelle ribonucleoproteiche non è influente sulla sopravvivenza degli embrioni, ipotizzerei che l'inserzione della GFP nel sito che ho selezionato possa essere letale, e quindi suggerirei di marcare il recettore in una posizione diversa.

Conclusione

Nonostante ci sia ancora tanto lavoro da fare, il mio progetto sarà alla base di un obiettivo più ambizioso: riuscire a filmare le cellule staminali posteriori (teloblasti) per l'intera durata della vita dell'individuo.

Il protocollo di modifica genomica che ho iniziato a sviluppare è un passaggio fondamentale per la marcatura fluorescente di proteine in *Platynereis*. Per l'acquisizione della maggior parte delle immagini che ho ottenuto, mi sono servito di microscopi confocali. Le tecniche di microscopia utilizzate sono soddisfacenti per le tematiche che ho già discusso. Tuttavia, la messa a punto di nuovi protocolli potrebbe consentire di visualizzare l'aggiunta postembrionale di segmenti *in vivo*. Questo progetto prende in considerazione l'utilizzo di microscopia a foglietto di luce e di dispositivi microfluidici. L'ottimizzazione di tutti questi protocolli permetterà per la prima volta l'osservazione di un pool di cellule staminali altamente multi potenti nella loro nicchia non perturbata. Sarà possibile utilizzare degli strumenti come i reporter del ciclo cellulare FUCCI per studiare come le perturbazioni di alcuni elementi della nicchia possano influire sul mantenimento delle cellule staminali e la tecnica BRAINBOW per tracciare una mappa precisa del destino di ogni singolo teloblasto. Dal momento che le masse tumorali sono unicamente iniziate, promosse e propagate da una piccola frazione di cellule staminali, definite cancer stem cells, e molto difficili da osservare, ritengo che *Platynereis dumerilii* possa presto emergere come un organismo modello alternativo per lo studio del cancro, per determinare come cambi l'attività di cellule staminali adulte altamente multipotenti, rispetto alla composizione dell'ambiente extracellulare.

Abstract

How did the common ancestor of Bilaterians look like is still a debated topic. The traditional theories claim that it was unsegmented, but new scientific evidences are opening the field to a revolution of this perspective. The aim of this work is to broaden the knowledge on segmentation of *Platynereis dumerilii*, an emerging model organism. The addition of segments relies on waves of gene expression regulated by signaling pathways in several species. In *Platynereis* segments are added at the posterior tip of a growing animal for its whole life and after the regeneration of the posterior tip. In both cases, the segments are originated entirely from a set of stem cells, called teloblasts. I performed some imaging from a FUCCI cell-cycle reporter to optimize its fluorescent signal. Furthermore, I screened the oscillatory behavior of some Notch potential effectors, such as Hes genes. Finally, I started to develop a protocol to fluorescently tag the Notch receptor with CRISPR/Cas9 knock in, since it is involved in posterior addition of segments both in arthropods and in vertebrates. This project is intended to lay the groundwork to a more ambitious purpose: allow the long-term imaging of multipotent stem cells in their undisturbed niche.

1. Introduction

1.1 Segmentation in Bilaterians

Segmentation is a striking morphological feature displayed by a great number of animal species. Bilateria is a clade that includes all the bilaterally symmetric triploblast animals, including Vertebrates and a wide majority of Invertebrates, that may be either segmented or unsegmented. The origin of the segmentation of the body plan in Bilaterians is still a controversial question that arose early among the scientific community. As Balavoine points out [1], the key question is the presence of a segmented antero-posterior axis in the common ancestor of Bilaterians, called “Urbilateria”.

The traditional theories [2] claim that Urbilateria was unsegmented, implying at least two cases of evolutionary convergence that made this trait arose independently in each phylum of segmented Bilaterians. In this case, the common ancestor of Bilaterians is supposed to resemble a non-segmented flat worm, like a Planarian.

On the other hand, some hypotheses [3] propose that Urbilateria looked more like an annelid, showing segmentation and a number of pre-existing organs, excluding the multiple cases of evolutionary convergence. Notwithstanding the simple appearance of the biological question, it still remains contentious for a number of reasons and the only way to inquire it is to compare segmentation mechanisms of different species.

One of the most common clues supporting the planarian-like Urbilateria is the profound difference between the definition of segments in distinct phyla. Indeed, the majority of species displaying a segmented anteroposterior axis are in the clades of Vertebrates, Annelids and Arthropods. The last two groups are showing a similar segmentation style, involving the segmentation of both ectoderm and mesoderm.

On the other hand Vertebrates are showing an evident and striking segmentation at mesodermal level that is only partially extended to the ectoderm. Such a profound difference is also confirmed at a cellular level, since in arthropods the segmentation is dictated by the ectoderm, while in the vertebrates the signaling centre has a mesodermal localization.

Therefore, arthropods and annelids have been traditionally classified as closely related organisms, basing on morphological features, in a superclade called "Articulata". This supported the idea that segmentation arouse twice in the evolutionary history of Bilaterians, displaying a different morphology.

The perspective is strengthened by the lack of knowledge on major genetic similarities in the segment formation processes.

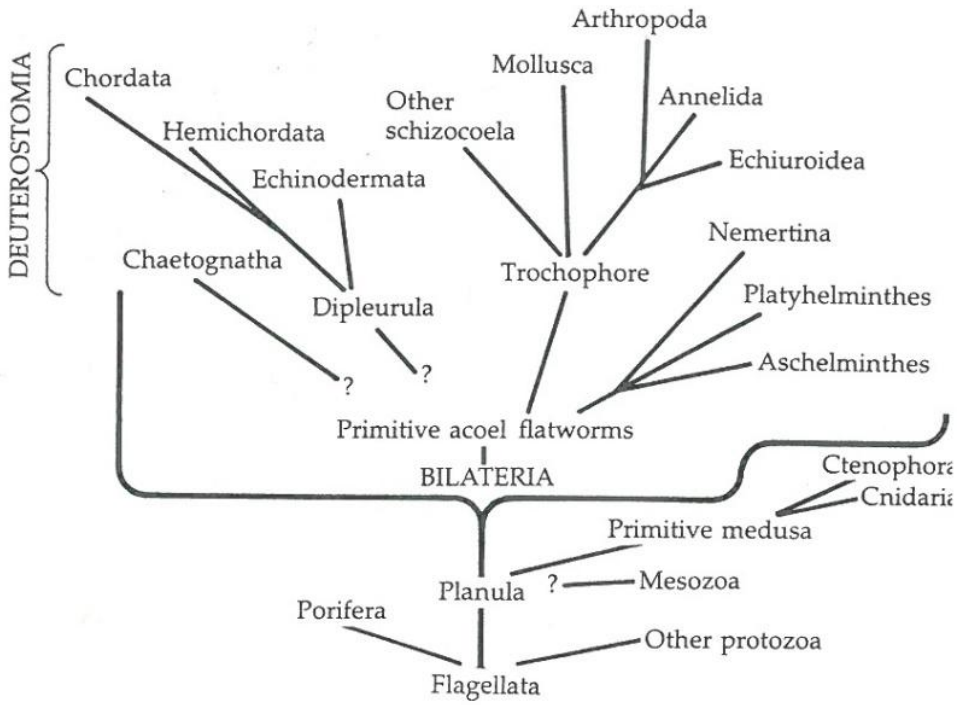

Fig.1 - A traditional phylogeny based on morphological features groups Annelids and Arthropods together, Brusca & Brusca, Sinauer, 1990.

On the other hand, more robust classifications were proposed in the late 90s by Aguinaldo [4], taking into account the genomic sequences, especially the ribosomal DNA. This turned out to be a breakthrough to determine that the "Articulata" superclade does not exist at all, Annelids and Arthropods being far apart in the Bilaterians tree.

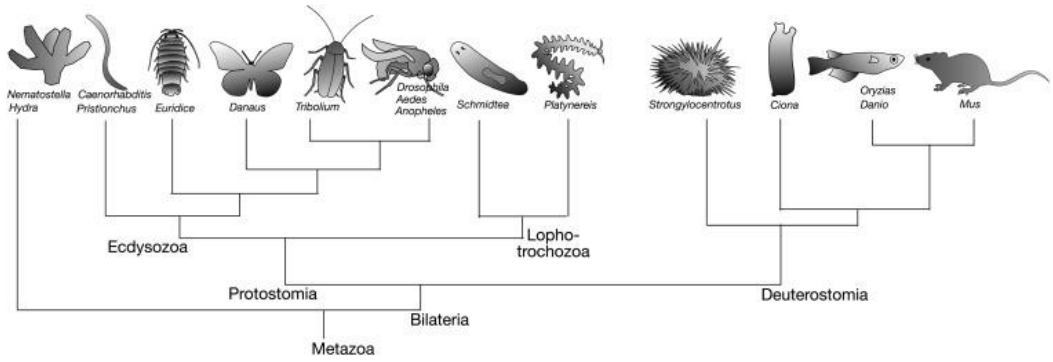

Fig. 2 - Tree of the model organisms, Zantke et al., Genetics, 2014.

Therefore, the origin of similarity between the metameric organization of Arthropods and Annelids turned out not to be so obvious. Additionally, a broader perspective on segmentation tends to consider segmented species that do not belong to the three previously mentioned clades; two examples are tardigrades and chitons.

Figg. 3 and 4 - Tardigrades and Chitons are segmented animals - pourlascience.fr; inaturalist.nz .

1.2 The segmentation clock

The segmentation of the body plan is therefore a common strategy in the morphogenesis of different species. As Richmond points out [5], it is not only limited to animals, but it also concerns the plant roots. The common ancestor of animals and plants was unicellular, therefore the two groups must be evolutionarily convergent; on the other hand, the presence of highly conserved molecular networks between Annelids, Vertebrates and Arthropods would be a strong proof of the segmented appearance of “Urbilateria”. In any case the question still remains opened because the pleiotropy of the involved pathways keeps the unsegmented Urbilateria scenario still potentially valid.

In vertebrates, the anteroposterior axis is sequentially segmented, with a species-specific period. The segmentation is dictated by a posteriorly sweeping dynamic wave which induces permanent modification in cell properties, either defined “clock” or “oscillator”. Masamizu’s work [6] in mice provides the evidence of cycling expression of Hes1 gene in dissected mesoderm by bioluminescent reporters, showing how this mechanism is cell-autonomous. Lewis J proposed a model to explain the molecular mechanism behind the oscillatory waves in *Danio rerio* [7]. Particularly, the production of the messenger of the Hes1 and Hes7 genes is repressed by their own periodic product. Segments are patterned at the wavefront, which is given by a gradient of factors. Particularly, perturbations of Wnt, Retinoic acid or Fibroblast Growth Factor are able to change the wavefront, distorting the shape of the somites [8] [9] [10]. The oscillations in isolate cells have been proven to be unstable [6], and the Notch pathway seems to be involved in the synchronization of neighboring cells [11].

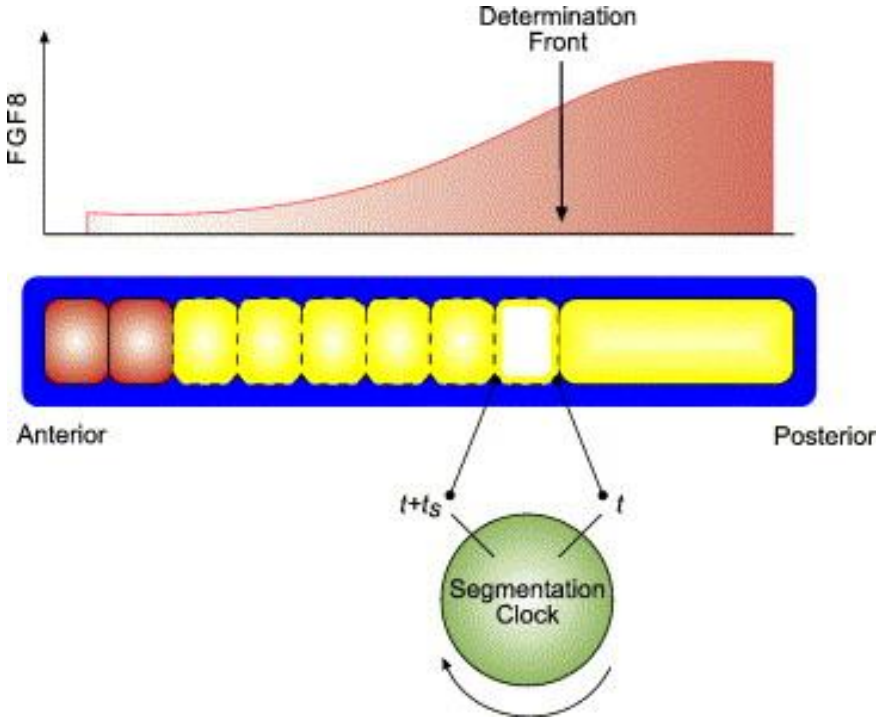

Fig. 5 - Baker *et al*. (2006) - The Segmentation clock and the wavefront in Vertebrates. An FGF8 gradient is specifying the position where the somites are formed. The segmentation clock produces waves that are attenuated at the wavefront, where the segments are patterned.

The majority of arthropods adds are adding segments posteriorly as the body plan elongates, with the major exception of *Drosophila*, where the segmentation is simultaneously dictated in a syncitial environment [12]. The existence of a segmentation clock is observed in the short germ Coleopteran *Tribolium castaneum* [13], relying on an indirect network of autorepression [14]. By far, the protocols on isolated cells or the use of bioluminescent probes are not yet developed in *Tribolium*.

1.3 *Platynereis dumerilii* an excellent species for comparative biology

In order to better interpret the segmentation among the Bilaterians it is also useful to broaden the study of segmentation on understudied groups. The position of the annelid *Platynereis dumerilii* in the phylogram of Bilaterians makes it to be an excellent species for comparative studies. This worm became a model organism for the study of evolution and development, environmental marine biology [15] and tissue regeneration [16], as well as evolutionary biology [17], since it is easy to manipulate and to breed in a laboratory with the possibility of obtaining embryos during the whole year. Both the embryos and the juveniles are transparent and this additional feature allows the use of confocal microscopy at any stage of development to study *in vivo* the conditional expression of proteins [18]. Albeit still not available on the databases, its genome has been sequenced and almost fully assembled, while transcriptomic data are accessible. Two main techniques of genome editing have been successfully tested on *P. dumerilii*: CRISPR/Cas9 and the Tol2 transposition system.

When it comes to morphology, the worm displays an outstanding metameric organization. Indeed, the only non-segmental parts of the juveniles and adult animals are the head and the terminal part, called “pygidium” who carries two tentacular appendices called “cirri”. The only difference between the anterior and the posterior segments is their size. Anterior segments, which are older than the posterior ones, tend to be bigger. An individual, according to its age, may have up to 80 segments.

Larvae are pelagic while the juvenile worm displays a benthic behavior, since it lives in tubes it produces with its silk glands. *Platynereis* are gonochoristic, and that means that male and female sexes are separated, and the sexual

dimorphism becomes clear a few week before the sexual maturation. Their sexual determination still remains unclear.

1.4 *Platynereis* is an ancestral species where pluripotent posterior stem cells named teloblasts are the responsible for segment addition and posterior elongation

P. dumerilii normal embryonic and larval development has been subdivided into 16 stages, easily identifiable with ordinary light microscopy, and it depends on the temperature, so it is possible to regulate the speed of this process. In order to set a reference, *Platynereis'* stages are described when development occurs at 18°C. After the fertilization, the zygotes display some lipid droplets, placed below the cortex, and start to produce a protective jelly. *P. dumerilii* displays a spiralian cleavage; the first cleavage occurs after the second hour post fertilization (hpf). Later it gives rise to a spherical ciliated larva, defined as "trochophore". As the development progresses, segments are generated in two steps. First the trochophore elongates to form the "nectochaete", simultaneously producing three early segments. The addition of segments to the posterior tip is a process that lasts the whole duration of *Platynereis*' life cycle; after the larval stage, segments are added from a pool of stem cells, named "teloblasts", located at the posterior pole of the individual, in a subterminal area called "Segment Addition Zone" (SAZ). A more exhaustive description of Platynereis' life cycle is portrayed by Fisher and Henrich [19]. The Annelid's teloblasts have been the focus of active research for several years. Not only they have been characterized by their multipotency transcriptomic signature (the Germline Multipotency Program, GMP), but also they have been proven to be the only responsible for segment addition in this species [20]. This discovery opens a rather new scenario considering possibility to live image for long periods highly multipotent stem cells, to describe their behaviour in terms of activation of signaling pathways, waves of mitosis or cell lineage.

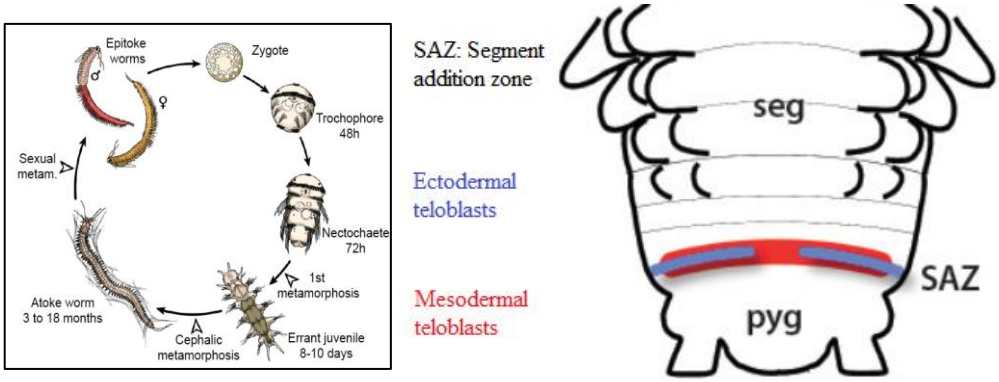

Fig. 6 - *Platynereis dumerilii*'s life cycle - A. Demilly, adapted from various sources; a scheme of the posterior region of a juvenile worm - G. Balavoine.

1.5 Wnt/beta-catenin and Delta/Notch signaling pathway and posterior addition in annelid and other models

The term 'Wnt signaling' is meant to cover a number of signaling pathways occurring at a cellular level. Particularly, three kind of Wnt signaling are well-known: canonical Wnt signaling, Planar Cell Polarity (PCP) Wnt signaling and the Calcium Wnt pathway. There are several Wnt ligands that can bind a Frizzled receptor and the biological signal is transduced through the protein Dishevelled. In the case of the canonical pathway, the activated Wnt pathway brings to the translocation of the β-catenin transcription factor to the nucleus.

Wnt signaling is proven to be involved in many developmental processes and in the control of cell cycle and proliferation. In *Platynereis dumerilii*, it is proven to be active at the posterior tip of the worm, suggesting a role in the proliferation of posterior stem cells.

Notch juxtacrine signaling pathway is a conserved biological process who allows neighbor cells to communicate. It is carried out through a transmembrane receptor of the Notch family, who binds a Delta ligand. When the pathway is active, the protein γ-secretase cuts the Notch Intracellular Domain (NICD), which acts as a powerful transcription factor, being translocated to the nucleus.

Notch is a crucial signaling pathway that is involved in a wide variety of biological roles such as neurogenesis and lateral inhibition both in insects and in vertebrates. The Notch pathway has also been explored in Lophotrochozoa, especially in *P. dumerilii* [21]. This study provides the unexpected evidence

that this pathway is not relevant in the general neurogenesis of larvae, while it seems to be involved in the outgrowth of the chaetal sac, the organ that produces the bristles.

1.6 *Platynereis dumerilii* in a laboratory

Storage

P. dumerilii has emerged as a good excellent model, representative species of Lophotrocozoan, due to the easy way to keep a culture in the laboratory and to obtain embryos almost every day of the year The complete protocol to keep *P. dumerilii* cultures in a laboratory is available at the website http://www.staff.uni-giessen.de/~gf1019/home/. It is bred since 1953 and in our research institute, two rooms are dedicated to *P. dumerilii*'s culture. In those rooms there are mechanisms to reproduce the sunlight and the moonlight, and it is important that the two rooms display an opposite moon cycle, in order to obtain embryos almost every day. Indeed, the moon cycle is essential to coordinate the breeding behavior and sexual maturation. Their temperature is kept at 18°C, since this species has a maximum peak of reproduction rate in spring. Oxygenation is needed worms that are younger than two months and in adults, since they can be sensitive to the drop of the oxygen level which may be due either to rotten food or to dead individuals. In my period at the Institut Jacques Monod, I have been involved in three procedures aiming to maintain our culture: transfer, feeding the juveniles and collection of the adults.

Transfer

Worms are stored in two different kind of boxes, containing 800 mL of natural seawater. The high-density boxes contain up to two hundred worms that tend to be smaller than the ones living in low-density boxes. Low-density boxes, also called "maturation boxes" contain 30-40 worms and are regularly made picking worms from high-density boxes, since worms do not mature at high density. Juvenile worms live in tubes, so they have to be chased with a pipette in order to make them leaving their tube before collecting them. This procedure is done once every two weeks.

Feeding

The worms are fed with different food according to their age. The youngest, between one week and one month old are fed with 5 mL of *Micro tetraselmis* algae, while the intermediate, between one and two months old are fed with

5mL of Sera Micron® 0,5 g in 50 mL of seawater. This procedure is repeated twice a week. Juveniles are fed with Tetramin® "fish flakes" and spinach. Worms are resistant to starvation for two weeks and it is important not to feed regenerating worms, to reduce their gut content which may contaminate the regenerating posterior ends that are going to be collected for the experiment.

Collection of the adults

Mature worms are stored in distinct boxes, accurately separated from the juveniles. Adult worms are identifiable by their pigmentation and the lack of their gut content. Males are red pigmented and females are yellow pigmented. In some cases, the pigmentation of the worm is not enough to tell a male from a female; it is a good habit to put ambiguous worms in the male box, since the presence of a male in the female box can determine the release of the eggs, and the subsequent death, of all the females. The adults' boxes are oxygenated.

1.7 Aim of the project: starting to characterize the segmentation oscillator of *Platynereis*

Some late processes in the establishment of the anteroposterior axis such as segment polarity have been proven to be regulated by conserved mechanisms such as the Hedgehog signaling [22]. Here, I lay the foundations for the quest of similarities among species in the first patterning events.

Imaging and optimization of a cell cycle fluorescent reporter (FUCCI)

The first aim of this work is to determine whether teloblasts oscillate in a coordinated way at a mitotic level during the addition of segments. The first observations of synchronized mitosis in teloblasts are highlighted by De Rosa, Balavoine and Gazave [20]. The Balavoine's team is now aiming to live image the dividing cells labeled with Fluorescent Ubiquitination-based Cell Cycle Indicators (FUCCI). I was involved in the imaging of the embryos to validate the efficiency of the constructs, proposing strategies to maximize the intensity of the three fluorescent signals.

Assess the oscillations of genes in coordination with the stem cells

The second aim of this work is to evaluate whether there is evidence of genes displaying an oscillatory behaviour at the posterior part of the worm while segments are added. This preliminary screening is essential to screen the genes that may be part of the oscillator in the Annelid. This would allow, together with the establishment of a proper knock-in protocol to select and target any

oscillating gene. From the literature, there are interesting suggestions that the gene caudal is oscillating in the Annelid [23], and I suspect that some of the HES genes may be oscillating too, accordingly to the pioneer work of Masamizu on mice [6] and the expression of several genes in teloblasts [24]. The Hes gene is a highly prolific family and *Platynereis dumerilii* has retained 12 Hes genes. As there is no clear homology between *Platynereis*' Hes genes and the ones from other model organism, I decided to focus on the ones that are expressed in teloblasts: Hes1, Hes2, Hes4, Hes5, Hes6, Hes8 [24]. Therefore, after I selected the Hes genes that are expressed in teloblasts, I performed a Whole Mount In Situ Hybridisation (WMISH) in regenerated tissues and in four weeks old larvae. Transmitted light microscopy and confocal microscopy was used to acquire 3D patterns of the expression of these genes. A precise screening of the stained tissues is required to find labeled or unlabeled tissues, incomplete rings or anything that makes us suspect an oscillatory expression.

Involvement of the Notch pathway in the molecular oscillator

I have been involved in the establishment of a CRISPR/Cas9 knock-in protocol, to develop a novel Notch reporter worm. The need of fluorescent reporter emerged because the in situ hybridization of the receptor is not informative, being it ubiquitously expressed. Moreover, the ambitious goal is to develop a whole life cycle reporter for signaling activity. Hence, the idea behind this conception of the reporter is to assess the activation of The reporter is conceived to tag the Notch Intracellular Domain (NICD) with EGFP, just before its stop codon. Notch pathway determining the localization of the fluorescent signal. Indeed, it is expected to be at the membrane when the pathway is inactive and to switch at a nuclear localization when γ-secretase cuts the intracellular domain to activate the signaling pathway. The establishment of both mosaic and germline fluorescent pathway reporters are milestones allowing to determine how Notch pathway is involved in posterior growth. Is it part of the segmentation clock? Is it synchronizing the oscillator of neighboring cells? Is it defining the segment polarity?

2. Materials and methods

2.1 Injection technique

Zygotes or 2-cells embryos are microinjected with recombinant DNA and genome editing proteins such as Cas9 and Tol2 to perform knock-ins. The activity of the microinjected proteins produces insertions of the recombinant DNA in some cells, producing mosaic animals. Mosaic animals can be already imaged, but to obtain permanent transgenic lines it is necessary to wait for the next generation, crossing animals labeled in the germ line. The microinjection technique needs training and experience. A trained worker is able to inject up to 150 embryos per injection. I have been training for three months and shared with Guillaume Balavoine the work of injections.

Fertilization

To obtain the zygotes to inject, I usually put three mature females and two mature males in an appropriate glass dish. It is possible to distinguish males from females by their pigmentation, since males are red and females are yellow. Mature worms are always showing a stereotypical way of swimming, which enables us to choose the fertile ones. It is important to fertilize more than one female, because some of them are laying eggs that will develop improperly and will not produce any living embryo. After a nuptial dance, the female discards 2000-3000 eggs, fertilized by the males' sperm. The zygotes are collected and incubated them at 18° for one hour, in order to let the jelly be completely secreted.

Fig. 7 - From left to right: a male, a female, properly developing eggs (cleaving), improperly developing eggs (uncleaving).

Construction of the platform

While waiting for the zygotes to develop, I build the platform for the injection, which is a bar of agarose melted in seawater with a trench of a conventional shape, held by a small Petri dish. First of all, 0.75 g of agarose are melted in 50 mL of seawater, filtered at 0.2 μm. To do this, there is a precise timing for the

microwave oven, where the mixture should remain 1.20 minutes. After that we shake the solution until any piece of agarose is melted, heating ten more seconds if it is necessary. We remind at this point that the mixtures of agarose and seawater are hard to be melted and big pieces will never dissolve back totally. Using a box, a special mold, a microscopy slide and some parafilm hydrophobic bands, the platform is made by pouring a thin layer of agarose all around this items. It is important to build the platform each time you inject, in order to avoid bacterial contaminations. The final shape of the platform is shown in the picture below.

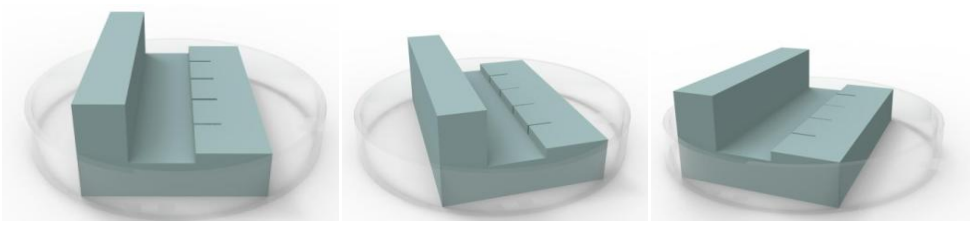

Fig. 8 - The injection platform

It is vital that the concentration of salts in platform is then equilibrated by pouring filtered seawater on it, since the melting in the microwave is always carrying out some extent of evaporation with it.

Removal of the jelly and softening of the cuticle

After one hour at 18°C, embryos are growing in a jelly produced by themselves. At this step it is important to remove it with ten washes in filtered seawater, performed with an appropriate filter 80 μm, after having mixed vigorously. This is followed by a quick treatment of proteinase K (18,75 μL in 30 mL of filtered seawater for 30 seconds) in order to soften their cuticle followed by ten more washes with filtered sea water. At this point all the jelly should be removed.

Setting up the microinjection platform

Firstly, all the electronic devices needed for the microinjection, such as the inverted microscope, the Femtojet and the manipulator are switched on. An additional stereomicroscope is needed to pipette the embryos to the injection platform. Then, the Femtotip needle is filled with two μL of the solution that is going to be injected, using capillaries. After the needle is ready, zygotes are transferred to the trench. It is important to remind at this step, that all the embryos must be injected, to avoid any bias due to the differential survival ratio between injected and non-injected embryos. Moreover, for the same

reason, it is important to transfer very gently the platform to the inverted microscope, in order not to disperse the embryos all along the entire platform.

Microinjection technique

Once the platform is transferred to the inverted microscope, it is possible to start the microinjection. The platform is placed on a sliding manipulator that allows the gentle motion of the platform without any vibration. First of all, it is important to look by naked eye at the needle and to put it in the middle of the light cone. The needle should then be plunged in the water, and the focus should be adjusted on the needle. This way, it is possible, changing the objective, to put the needle at the same level of the zygotes, slightly varying its height together with the focus. Once the needle is in the focus, at the same level of the zygotes, it is possible to push the developing embryos towards the high cliff, that should remain on the left. Before starting injection, it is crucial to verify whether the needle is clogged. When that happens, it is possible to unclog it by rubbing it on the big cliff or using the "flush" which is a powerful ejection of liquid from the needle. More precisely, the injection is possible due to the Femtojet, linked to the needle and the input is given by a mouse. The left click is the regular injection, while the right click is the flush to clear the needle's tip. Embryos are pushed to the high cliff and are pricked placing the needle at a precision position, that is easy to determine playing with the focus plan. Particularly, when the needle is able to push the zygotes it is on the same plan of the embryos, whose cuticule appears to be translucent. When the embryo is close to the high cliff, it is important to rotate the small focusing wheel by a quarter of a turn, slightly raising the focus plan. This way, it is possible to lift the needle until its tip appears clearly in focus. The platform is pushed to the immobile needle. The injection of few picoliters of solution is done by a left click of the input mouse. After the injection, the embryo remains pricked on the needle. To get rid of it, it is possible to scratch the needle in a cut made on the small cliff. The embryo is pushed down to keep track of the already injected embryos. This procedure is repeated for all the embryos. Embryos are collected and they are stored in a 18°C incubator to let them grow.

2.2 Imaging and optimization of a cell cycle fluorescent reporter (FUCCI)

The FUCCI technique is used to reveal the cell cycle progression. It is possible to inject the reporter as a messenger mRNA, or to knock it in *Platynereis'* genome. A suitable tricistronic FUCCI mRNA would be the breakthrough to

study the cell cycling at the beginning of the Annelid's early development, but its expression drop quickly. On the other hand, mosaic individuals are not the best strategy to reason on that question, since the genome is starting to be expressed only just after the maternal-to-zygotic transition and are more suitable to study later stages. Since the expression of this mRNA is not yet optimized in *Platynereis* and the CRISPR/Cas9 protocol is not yet well developed, I decided to test the construct developed by Balavoine's team co-injecting the plasmid with a Tol2 transposase. This produces random insertions in the genome. Therefore, some embryos are expected to be mosaic.

Structure of the chimerical DNA

The chimerical sequences coding the FUCCI proteins produced by the Balavoine's team are inserted in a plasmid containing the Tol2 inverted repeats to make the transposition of the DNA possible. The insert should a tricistronic messenger, with a separate expression of two cell-cycle markers and a nuclear protein. More in detail, the protein Cdt1, marker of the phases G1 and G2 of the cell cycle, is fused to a green fluorescent protein, the histone H2A is fused to the fluorescent protein mMaroon1 to label the nuclei at any stage of the cell cycle and, the cyclinA, reporter of the late G2 phase, is tagged with mKO2, an orange fluorescent protein.

The three chimeric structures are separated by 2A ribosome skip sites. These sequences should be able to separate the three different proteins. Their transcription is promoted by the site rps9 (Ribosomal Protein S9 promoter), who is ubiquitously expressed from 15 hpf.

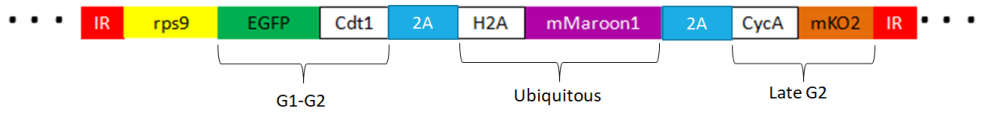

Fig. 9 - The FUCCI construct.

Injected solution

The following table shows the exact composition of the injected solution:

Reagent	Concentration
pTol FUCCI A	30 ng/µl
capped mRNA Tol2	100 ng/µl
Red Phenol	1/15

Observation

Overnight movies of fluorescent embryos are acquired overnight using the confocal microscope LSM 710 Zeiss. The embryos are mounted in agarose melted in seawater, stocked at -20°C and melted at 42°C each time it is used. While mounting the embryos, the plate is kept at 37°C. It is important to make sure that the embryos are at the bottom of the plate, in order to guarantee proper imaging.

2.3 Oscillatory behavior of Caudal and HES mRNA in teloblasts

I suspect that the posterior addition of segments relies on the oscillatory expression of some genes. Segment addition in the annelid occurs slowly during the whole life cycle of the animal, but gets accelerated after posterior regeneration. The whole process of regeneration takes about one week and it is rather variable across different individuals. After the regeneration of the pygidium, the addition of segments starts. So, a seven-day post amputation (7 dpa) population of worms provides a wide range of tissues at different time points after the start of regeneration, where the majority of worms have entered the phase of segment addition. As highlighted by A. Planques *et al.* [16], the process of the regeneration occurs in five reproducible steps, as in the picture:

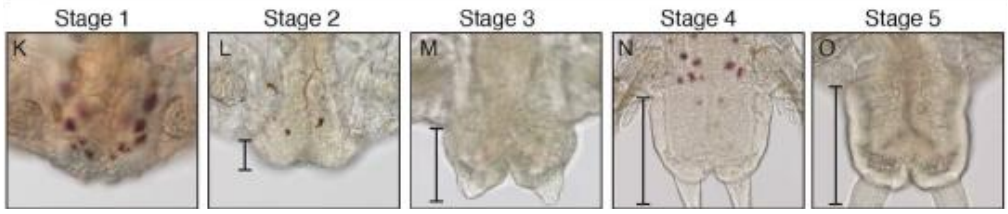

Fig. 10 - Planques A, *et al.* The stages of regeneration in *Platynereis dumerilii*: 1) Wound healing 2) Small regenerate with anus reformation 3) Elongation of the regenerate and cirri regeneration 4) Size of the regenerate and cirri length are increased 5) An indentation separates the anterior part of the regenerate from the posterior. After this stage, segment addition starts.

By the way, this experiment does not guarantee the reconstruction of the dynamics of the messenger production, due to the limited number of samples. For this reason, I decided to extend the experiment to a broad population of juvenile 4 weeks old worms. The assessment of differential transcription of mRNAs relies on the hybridization of probes of the genes: Caudal, Hes1, Hes2, Hes4, Hes5, Hes6, Hes8 on fixed samples. The probes are labeled with

Digoxygenin, recognized by an antibody bound to an alkaline phosphatase (AP). The alkaline phosphatase reacts with Nitro blue Tetrazolium and 5-Bromo-4-Chloro-3-Indolyl phosphate producing a blue dye in the tissue where the gene is transcribed. Samples are cleared in TDE and mounted on microscopy slides.

Preparation of the solutions

Before the beginning of the experiments, the following solution are prepared:

1) Stock of Paraformaldehyde 16%.
2) One liter of PBT (Phosphate Buffer Saline, PBS 1x + 0.1% Tween-20)
3) TH buffer (for 50 mL: 25 mL of Formamide, 12.5 mL of SSC 20x pH 7.5, 125 mL of Heparine 50 mg/mL, 250 mg of Torula RNA powder, water to 50 mL).

Day 1: Fixation of juveniles

1) Four-week old juveniles are collected and put in 15 mL Falcon © tubes.
2) A tube of Paraformaldehyde (PFA) 16% is thawed for 10 minutes at 60°C or until it is entirely dissolved. This tube may be used up to fifteen days after this step, if stored at 4°C.
3) «Prefix» solution is made mixing 3 mL of PFA to 6 mL of PBT 2x
4) The juvenile worms are washed once in «Prefix».
5) «Fix» solution is prepared mixing 4mL of PFA to 8 mL of PBT 1x
6) The juveniles are fixed overnight at 4°C or for 2 hours at room temperature
7) The biological samples are progressively dehydrated in PBT 1x for 5', in methanol 50% PBT for 15' and finally in methanol 100% for 1 hour at room temperature or overnight at 4°C.
8) Methanol is replaced and the samples are stored at -20°C

Day 1: collection and fixation of the regenerates

The first amputation of the tail is performed one week before the first round of collection of regenerating tissues. One hundred juvenile worms, 3 cm long, enough to be easily cut, have been collected and anesthetized in $MgCl_2$ 7.5% 50% in sea water to have their tail amputated with a sharp and precise cut between two distinct segments. After one week, the regenerates are collected and are fixed according to the following procedure.

1) A tube of Paraformaldehyde (PFA) 16% is thawed for 10 minutes at 60°C or until it is entirely dissolved. This tube may be used up to fifteen days after this step, if stored at 4°C.
2) «Fix» solution is prepared mixing 4mL of PFA to 8 mL of PBT 1x
3) The regenerate is collected together with three non-regenerated segments.
4) The tissues are fixed overnight at 4°C or for 2 hours at room temperature
5) The samples are progressively dehydrated in PBT 1x for 5', in methanol 50% PBT for 15' and finally in methanol 100% for 1 hour at room temperature or overnight at 4°C.
6) Methanol is replaced and the samples are stored at -20°C

Day 1: Synthesis of the probes

The probe should be inserted in a plasmid containing the transcription sites T7 and SP6. It is important to know the direction of the insert and to start from a concentration of 1.5-2 ng.

1) Linearize the fragment using a restriction enzyme of the polylinker that does not cut the insert. Use a volume, depending on the concentration of the plasmid, to have 2 µg of plasmid. The buffer 10x volume is adapted to the amount of plasmid. Digest with 1 µL of enzyme. The reaction takes place at 37°C for 1 hour in a total volume of 50 µL.
2) The results are verified by electrophoresis on agarose gel 0.8% and evaluated by nanodrop.
3) The sample is purified using the Qiagen Kit "PCR purification"

The probe is synthesized setting up the following reaction:

Linearized DNA (1-2 µgrams) + sterile water	14,5 µL
Protector RNase inhibitor (Roche)	0,5 µL
Dig/Fluo RNA labeling mix (Roche)	2 µL
Transcription buffer 10X (Roche)	2 µL
RNA polymerase T7 or SP6 (Roche)	1 µL
Final volume	20 µL

Mix and centrifuge briefly, then incubate two hours at 37°C. After one hour, add 1 µL of enzyme T7 or SP6.

Removal of the plasmid

1) Add 66,5 µL of RNase-free water, 10 µL of buffer and 2,5 µL of DNAase 1 for 15 minutes at 37°C.
2) Purify using the kit RNeasy (Qiagen)
3) Assess the concentration using Nanodrop
4) Resuspend in TH and store the probe at -20°C.

Transmitted light pictures are acquired to assess the differential transcription in tissues, while confocal microscopy pictures are acquired to have a more precise insight on the nature of the cells where the gene is transcribed.

Day 2: Rehydration

From the second day on, the instructions are given both for the regenerates and the juveniles. The differences between the two protocols are highlighted when it is needed.

Rehydrate the samples putting them in basket and transferring them following the instructions:

1) 5' + agitation in 75% MeOH + PBT
2) 5' + agitation in 50% MeOH + PBT
3) 5' + agitation in 25% MeOH + PBT
4) 5' + agitation in PBT
5) 5' + agitation in PBT

Day 2: Proteinase K digestion

To prepare the solutions needed for the Proteinase K digestion:

1) Thaw PFA 16% at 65°C
2) Thaw Glycine 10x at 37°C

Soften the cuticle of the worm treating them with Proteinase K. Glycine is a powerful inhibitor of Proteinase K.

1) For the regenerates, a Proteinase K 100 ug/mL in PBT for 3 minutes without agitation is recommended.
2) Juveniles have been treated with Proteinase K 1000 ug/mL in PBT for 10 minutes without agitation. As it is clear from the results, the protocol did not work. This is the first time that the *In Situ* Hybridization is performed on 4-week juveniles, therefore the protocol must be adapted.
3) A brief wash (30'' - 1') is performed In PBT Glycine 1x without agitation.

4) 20’ + agitation in PBT PFA 4%
5) 5’ + agitation in PBT
6) 5’ + agitation in PBT
7) 5’ + agitation in PBT
8) 5’ + agitation in PBT
9) 5’ + agitation in PBT

Day 2: Hybridization

Transfer the material in smaller baskets that are fitting a 2 mL Eppendorf © tube, filled with PBT. The following passages are always performed on a stock of re-usable 2 mL Eppendorf © tubes, accurately labeled and washed every time they are used. The samples are in baskets that can be easily transferred from a tube to another one with the help of forceps.

1) Denaturate the probe in TH buffer, 10’ at 80°C
2) Pre-hybridization: transfer the baskets in 400 uL of TH buffer for 1h 30’ at 65°C
3) Transfer the basket in tubes containing 200-300 uL of denatured probe, pre-warmed at 65°C in a water bath.
4) The samples are transferred to the oven, at 65°C for the whole night, at least for 16 hours.

Day 3: Washes of the probes

Once the probes are hybridized, it is important to wash the eccess of probe, keeping the temperature constant. For this reason, the following washes are performed on pre-warmed solution at 65°C in the water bath, subsequently transferred to a 65°C oven. It is important to do the passages very quickly, in order to preserve the temperature.

1) 30’ in SSC 4x Tween 20 0.1% 50% formamide.
2) 30’ in SSC 4x Tween 20 0.1% 50% formamide.
3) 15’ in SSC 2x Tween 20 0.1% 50% formamide.
4) 15’ in SSC 2x Tween 20 0.1% 50% formamide.
5) 30’ in SSC 0.2x Tween 20 0.1% 50% formamide.
6) Overnight 4°C in SSC 0.2x Tween 20 0.1% 50% formamide.

Day 4: Antibody incubation

The hybridized probes are captured by an antibody alpha-Dig-Alkalyne Phosphatase who recognizes the Digoxygenin.

1) Samples are transferred for one hour + agitation in 1 mL PBT + Sheep serum 5%
2) Meanwhile, the antibody Dig is preincubated in PBT + Sheep serum 5%
3) The antibody is incubated overnight at 4°C + agitation on samples, in PBT + 5% Sheep serum + 1/4000 antibody.

Day 5: Antibody washing

Samples are washed following the instructions:

1) 5' + agitation in PBT
2) 5' + agitation in PBT
3) 5' + agitation in PBT
4) 10' + agitation in PBT
5) 20' + agitation in PBT
6) Overnight 4°C + agitation in PBT

Day 6: NBT/BCIP staining

Samples are stained following the instructions:

1) 5' + Agitation in PBT
2) 5' without agitation in the pre-staining solution without magnesium chloride (Tris Cl buffer pH 9.5 0.1M; NaCl 0.1 mM Tween 0.1%)
3) 5' without agitation in the pre-staining solution with magnesium chloride (Tris Cl buffer pH 9.5 0.1M; NaCl 0.1 mM Tween 0.1% $MgCl_2$ 50mM)
4) Staining solution (made adding 3.5uL of BCIP and 4.5 uL of NBT to each mL of pre-staining solution with $MgCl_2$)
   The staining may last from few hours to several days. It is very important to change the staining solution after 24 hours of staining. Once the blue precipitate is well produced, it is possible to stop the staining with the stop solution (Tris Cl buffer pH 7.5 0.1M; NaCl 0.1 mM Tween 0.1%).

Day 7: Clearing and observation

Samples are stained following the instructions:

1) 5' + Agitation in PBT
2) 5' + Agitation in PBT

3) 5’ + Agitation in PBT
4) 10’ + Agitation in 67% PBT 33% TDE
5) 10’ + Agitation in 33% PBT 67% TDE
6) Overnight + Agitation at 4°C in 97% TDE

Embryos are put on microscopy slides covered by a coverslip. The regenerates must be oriented either dorsally or ventrally with the help of a thin hair. Juveniles are randomly spread on the coverslip. Transmitted light microscopy is used to reveal the differential transcription of the gene in different tissues, while confocal microscopy and 3D reconstructions are more suitable to detect interruption of expression in the ring of teloblasts.

2.4 Involvement of Notch pathway in the molecular oscillator

General strategy

The third aim of the work is to tag the Notch receptor by a green fluorescent protein, just before its stop codon. To do that, a CRISPR/Cas9 knock-in has to be performed for the first time in *Platynereis dumerilii*. Therefore, I built a recombinant plasmid, inserting EGFP just after Notch’s last exon. To have a broad region of homology to make the knock-in efficient and specific, I inserted the 2000 kb region surrounding the Notch stop codon in the PCR2 plasmid. The EGFP sequence is inserted just after the Notch last exon by Gibson Assembly, separated by a short linker. Since the whole insert is more than 3 kb long, I decided to assemble it by myself, to reduce the costs of the DNA synthesis.

Fig. 11 - The structure of the Notch Reporter

The recombinant single stranded DNA is synthesized following a protocol based on lambda exonuclease and coinjected with the Cas9 system to perform the tagging of Notch Intracellular Domain. Single stranded DNA has been proven to be more efficiently integrated in the genome in some studies, following the technique Easi-CRISPR [25].

Production of the chimerical DNA: Nested PCR, Gibson Assembly

The 2 kb region surrounding the Notch stop codon is amplified by nested PCR from *Platynereis*’ genome. Nested PCR is a highly sensitive technique to

amplify genomic regions without non-specific contaminants. It consists of two tandem PCR performed on a genome with two different couples of primers, partially overlapping one another. The primers are designed in order to avoid polymorphisms. Once the first PCR is performed, the mix is directly diluted 1:100, in order to reduce significantly the concentration of the first couple of primers, and a second round of PCR is performed with the nested primers, like in the following picture.

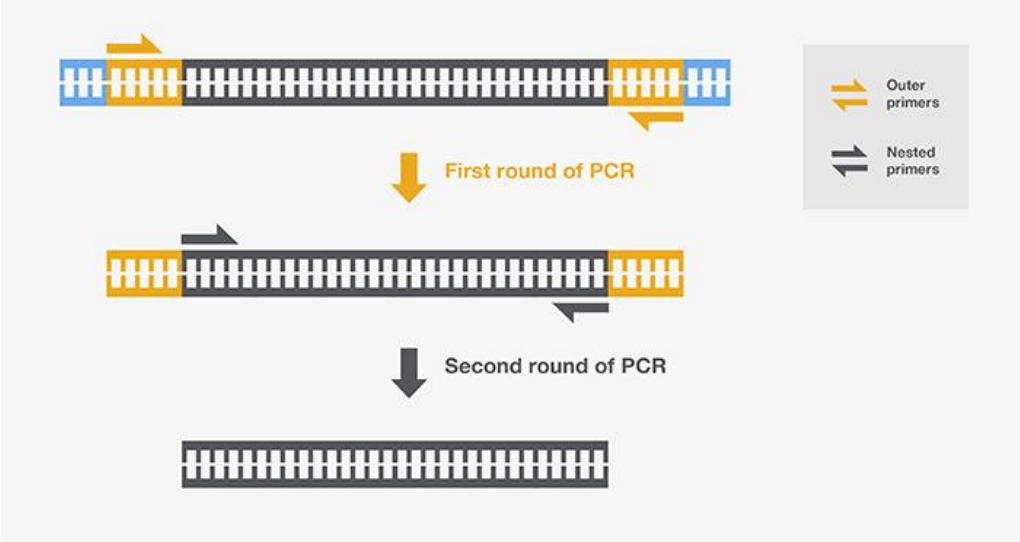

Fig. 12 - PCR methods - Top ten strategies - Thermo fisher scientific; thermofisher.com

The product is inserted into a PCR2 plasmid by Gibson Assembly and it is cloned in *Escherichia coli*. Gibson Assembly is a technique used to fuse overlapping fragments of DNA. Particularly it relies on a cocktail of enzymes with an exonuclease, a polymerase and a ligase.

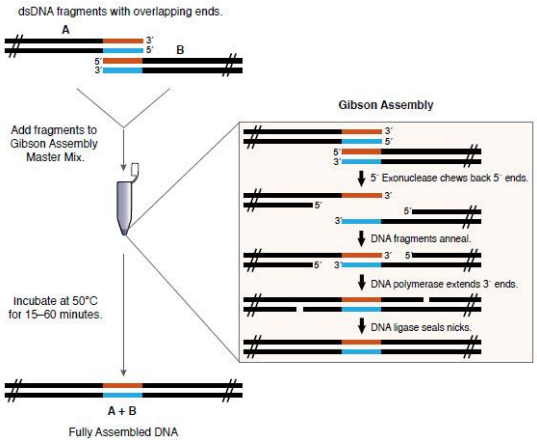

Fig.13 - The Gibson Assembly technique

Despite the lower rate of errors of the Taq polymerase used for the amplification, it is important to screen a good number of colonies Sanger Sequencing. The plasmid corresponding to the expected sequence is amplified in liquid culture and recovered by midi-prep. Enhanced Green Fluorescent protein (EGFP) is inserted in the selected site again by Gibson Assembly.

Since the assembled plasmid is used to do a CRISPR/Cas9 knock-in, at this step it is important to make sure that the Protospacer Adjacent Motif (PAM) sites in the plasmid are mutated, modifying the primers without changing the aminoacidic sequence. The Cas9 Protein is able to cut the genomic DNA in a very precise way, when it is directed to the genome through a specific guide RNA (gRNA). In order to cut, the Cas9 protein needs a NGG sequence on the target DNA, just downstream the cut site the PAM site. An efficient knock-in implies both an efficient cut on the wild-type genome and the blockage of this capability in the recombinant DNA. For this reason, the PAM sites on the plasmid are mutated, inserting synonymous mutations on the primers used to amplify the plasmid, during the phase of EGFP insertion.

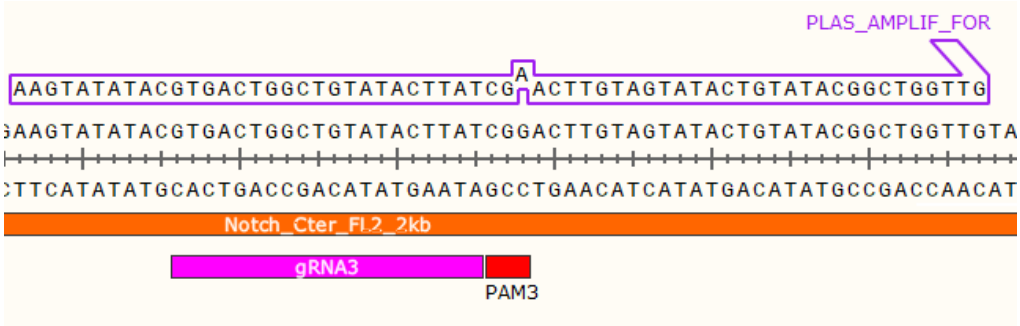

Fig. 14 - Mutation of a PAM site introducing synonymous mutations

To perform the cuts on the genome, a suitable mix of gRNAs cutting in proximity of Notch carboxyterminal domain is designed and tested both in *in vitro* on the extracted plasmid and in vivo to test the optimal concentration to inject. The *in vivo* test is expected to produce mosaic individuals displaying either deletions or insertions at the targeted region, following the DNA repair by Non Homologous End Joining (NHEJ).

Single stranded DNA synthesis

To produce the chimerical ssDNA, I used a protocol based on PCR and lambda exonuclease digestion. Particularly, DNA is amplified using a 5'phosphate primer and a 5'phosphorothioate primer. The 5'phosphorotioate end prevents DNA digestion by lambda exonuclease.

Duplex formation

I performed the injection with the CRISPR/Cas9 NEB enzyme and the gRNAs produced by IDT DNA. Since the transactivating crRNA (tracrRNA) and guide RNA (gRNA) are distinct molecules, it is important to anneal them. To do that, an equimolar solution of tracrRNA and gRNA is heated at 95°C. The tracRNA is dissolved in water, while the gRNA is dissolved in Hepes buffer. To do the knock-in we used an equimolar cocktail of three gRNAs.

Ribonuclear particles formation and in vitro test

To assess the functionality of Cas9 protein the gRNAs, an in vitro test is set up. The table on the left explains how to make the Cas9/gRNA complexes. The table on the right resumes the solution to make the in vitro test.

	Complex formation
Water	1.5 μL
gRNA	1 μL
Buffer KCl	0.5 μL
Cas9	2 μL

	In vitro test
Water	5 μL
Linear DNA	1 μL
Buffer KCl	1 μL
Cas9 complex	1 μL
$MgCl_2$	1 μL
Cut Smart buffer	1 μL

After having set up the last reaction, it is incubated for 30' at 37°C. Subsequently, 2 μL of Protein Kinase (PK) are added to degrade Cas9 that modifies migration on the gel. Thirty more minutes of incubation are needed. The result of the digestion made separately with the distinct gRNAs is loaded on a 1% Agarose Gel. Linear and circular plasmid are loaded as a control. A functional Cas9 complex should display two distinct bands, meaning that the protein executed its cut.

Mortality controls

Firstly, an injection with buffer 1x only is used to determine the mortality due to the mechanical stress produced by the microinjection, compared to non-injected embryos. Secondly, an high concentration of DNA in the cytoplasm can be to some extent toxic for the cells. Thus, before going on with the experiment I checked that the injection of 10 ng/μL is not toxic for the embryos. Some of the embryos injected with ssDNA are first deciliated, using a solution of DMSO in 10% water for 10 minutes followed by a flush of

natural sea water and mounted on glass bottoms, embedded in a droplet of low melting agarose 1% in seawater, to check at the confocal microscope LSM 710 Zeiss whether the ssDNA can be expressed when it is not recombined.

In vivo test

The Cas9-gRNA complex is then tested in vivo. A first test using the Cas9 complexes at 15 μM does not produce any normally developing embryo after 24 hours. Therefore, I kept decreasing the concentration of the injected solution up to 2 μM. A control of non-injected embryos is kept to assess the quality of the batch of eggs, which is naturally variable. After 24 hours, the genomic DNA is extracted from the injected embryos using the Phire Tissue Direct ® kit. At that stage, the embryos are displaying different behaviours. Looking at the binoculars, some of them are embryos are "swimmers" and they are the only ones that may develop correctly. Some others are "spinners", since they rotate on their axis. Last, among the immobile embryos it is possible to tell the ones that do not display any clear sign of decomposition.

The injection of a Cas9 complex without any knock-in cassette is expected to produce mosaic mutations in the injected embryo. That depends both on the activity of the Cas9 complex and of the capability of the organism to repair its DNA by non-homologous end joining (NHEJ). This is expected to introduce some small insertions or deletions in the DNA.

To determine whether the Cas9 is cutting in vivo, a genotyping strategy was followed. Particularly, a region of DNA 200 bp long is amplified around the cut site. The control embryos should show a single band of the controls in an electrophoretic run on a 2% low melting agarose gel. On the other hand, we expect the injected embryos to show small insertions or deletion, so the migration should appear like multiple bands or a smear. Swimmers, spinners, immobile embryos are selected, since the survival of the embryos may be hindered by this test.

After the microinjection: observation of the injected animals

The embryos injected with single stranded DNA only should not express the EGFP. This control is needed to claim that the ssDNA is not expressed if it is not permanently integrated in the genome. Some of the embryos injected with the knock-in cassette are expected to show patches of fluorescence. Snaps are taken at the confocal microscope LSM 710 Zeiss.

Additional genotyping controls

Once fluorescent mosaic lines are established, it is important to prove that the fluorescent protein is inserted in the right genomic contest. To do that, another couple of primers is designed. In this case, one of the primers is pairing with the EGFP exogenous sequence, and the other one lays out of the 3' UTR that was used to do the knock-in, to avoid any residual ssDNA amplification.

3. Results and discussion

3.1 Imaging and optimization of a cell cycle fluorescent reporter (FUCCI)

I mounted several embryos on a glass bottom. I selected three fluorescent mosaic embryos and I acquired the whole Z-stack of each embryo in multiposition mode, with an interval of 10 minutes for the whole night. When dealing with live embryos it is important to reduce phototoxicity. Thus, after a period of exposure to the laser, it is crucial to interrupt the exposure on the embryo for at least the double of the time it was exposed. Meanwhile it is possible to continue the acquisition of other positions. The following picture resumes a 90 minutes movie of a mosaic embryo that integrated successfully the FUCCI DNA in its genome.

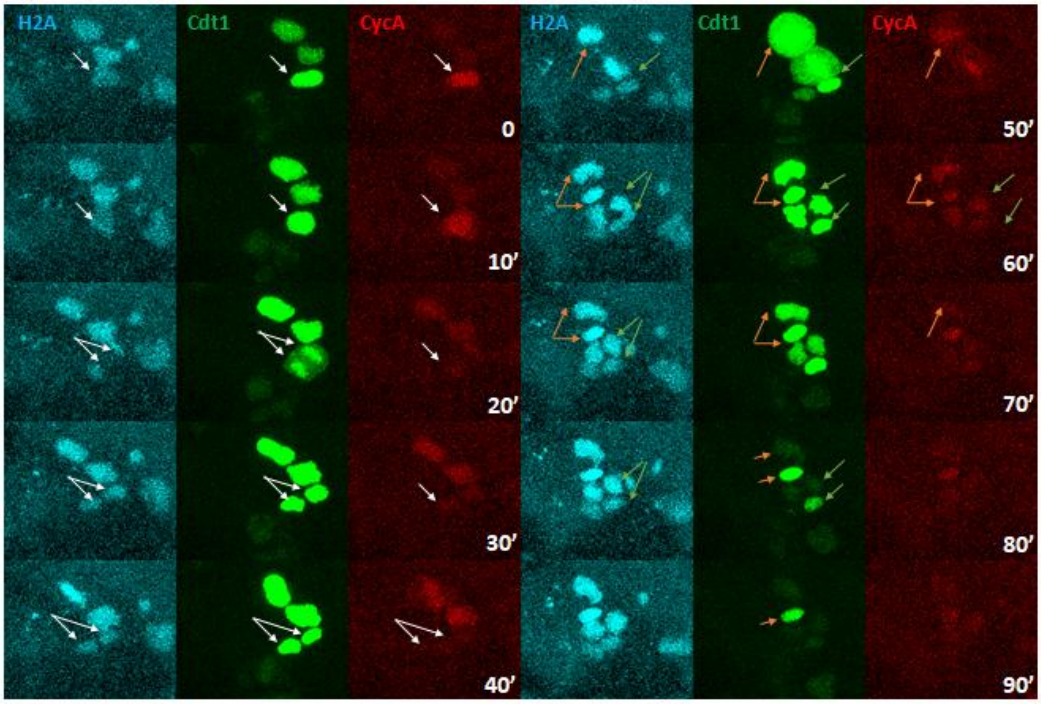

Fig. 15 - A FUCCI cell cycle reporter embryo; from 15 to 16 hpf magnification 40x. Cyan marks ubiquitously the nuclei; Green highlights the phases G1 and G2; Red labels the late G2 phase.

The picture convincingly shows that the FUCCI reporter is working well. An important milestone reached in this experiment is that the three channels are independent, meaning that the ribosome skip sites are working properly, producing three distinct proteins.

At the tenth minute, cell pointed by a white arrow is in the phase G2 and the bright red spot shows how it is expressing the Cyclin A. At the twentieth minute, the cell undergoes division. The two daughter cells, entering the G1 phase, are still expressing the protein Cdt1, while the red signal fades immediately. Similarly to the previous example, at the fiftieth minute, the cells pointed by green and orange arrows are in the phase G2 and expresses Cyclin A. After they divide, the Cyclin A is immediately disappearing, while the protein Cdt1 still is expressed, until the daughter cells enter the S phase. Here is evident how the four cells behave differently: two of them are immediately entering the S phase, and the two others are displaying a longer G1 phase and their green signal is more stable.

The FUCCI reporter still presents some problems. First of all, the signal of two channels is still extremely weak. To obtain a better image, it is necessary to enhance the digital gain and that produces a lot of background signal. Hence, in my opinion, the second and the third protein in the construct are expressed at an insufficient level and there is the need to optimize this construct.

The reason of the low signal of the two protein must be an inefficient restart of translation after the ribosome skip site. It is possible to overcome this problem following two different strategies. One solution can be the use of more efficient "new generation" ribosome skip sites. Otherwise, the order of the different proteins may be relevant too. For this reason, I would like to assemble the construct again, putting the less bright fluorescent in the first place after the promoter, which is mMaroon1, to be more efficiently produced, as exhaustively portrayed by Liu [26].

Another important step forward would be to make a tricistronic mRNA to test the involvement of mitotic waves in the early development of the embryo, because the rps9 promoter is never expressed before 15 hpf, when the maternal-to-zygotic transition happens. To do that, it is important to clone the FUCCI reporter in a proper context, an optimized expression plasmid with 5' UTR and 3' UTR of a highly expressed *Platynereis* gene.

3.2 In situ hybridization and oscillation of Cdx and Hes mRNA

Juveniles

The *in situ* hybridization gave powerful insights on the choice of potential candidate genes oscillating in teloblasts. In situ hybridizations on regenerates are performed to assess the differential localization of mRNAs. In order to make statistics on a larger number of samples, I started to do the *in situ* hybridization also on juvenile worms. Since my aim is to study segment addition, one month old worms are needed. Unfortunately, the *in situ* hybridization of one month old worms was never tried before and I had to make several tests to refine the protocol for my needs. Particularly, I determined the suitable duration of the Proteinase K treatment and a proper Proteinase K concentration to make the cuticle of the juvenile worms efficiently permeable by the probes. Since the development of the worms is synchronous in the first phase of their development but becomes desynchronized from the time the worm starts to feed (10 dpf), it is important to show that all the phases of the addition of segments are well represented in the population. Therefore, I counted the number of segments 4-week-old juvenile worms. In the following graph, the red dashed lines are separating the early segment formation from the late segment formation.

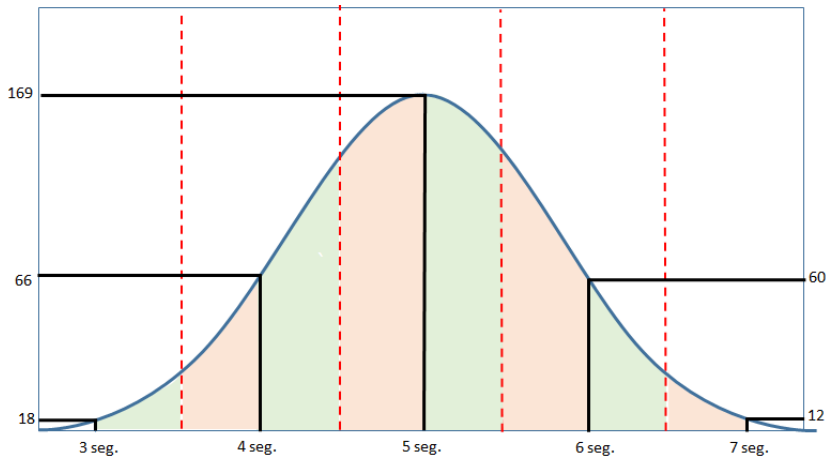

Fig. 16 - X: number of segments; Y: number of worms with a given number of segments, real data; black lines: counts of worms with a given number of segments; red lines: border between the early phase and the late phase of segment addition; green area: early phase of segment addition; red area: late phase of segment addition. The population is fully randomized and any stage of segment addition is equally represented.

Post caudal regeneration elongation

The following pictures are acquired at the transmitted light microscopy and elegantly show the patterns of caudal in one week regenerates. The labeling of the teloblasts is almost always showing incomplete rings; the pygidium is not always labeled in the same way. Sometimes only the very posterior part is stained, in other cases the whole organ appears blue. The early regenerates are sometimes presenting a peculiar striped patterning. On the ventral side, neurons are often labeled.

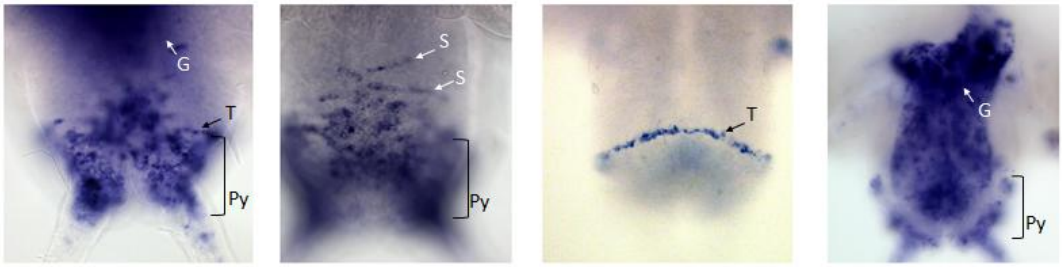

Fig. 17 - A selection of dorsal pictures, Cdx mRNA. (1) pygidium (Py), Teloblasts (T) and Gut (G); (2) Pygidium (py) Stripes in segmental ectoderm (S); (3) The ring of teloblasts (T) (4) Posterior pygidium (Py) and gut (G).

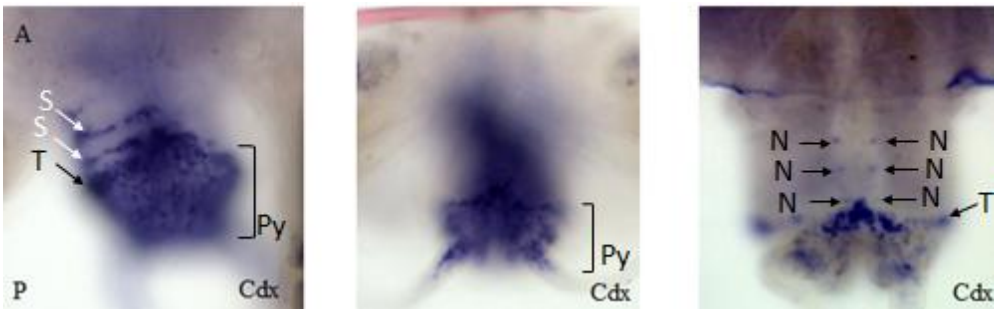

Fig. 18 - A selection of ventral pictures, Cdx, 20x, Ventral view. (1) Stripes in the segmental ectoderm (S), Teloblasts (T) and pygidium (Py); (2) Pygidium (Py); (3) Teloblasts (T) and Neural cells (N).

The following pictures are acquired at the transmitted light microscopy and elegantly show the patterns of Hes2 in one week regenerates. The dynamics for Hes2 is much simpler, since it only stains teloblasts and chaetal sacs. Chaetal sacs are constantly labeled by Hes2, therefore I used the chaetal sac staining as an internal control to asses that the *in situ* hybridization works. This is meaningful because some of the Hes2 are not labeled in teloblasts. If the chaetal sac is labeled, it means that reaction worked and the samples with

unlabeled teloblasts are not expressing Hes2 at all, being an exceptional example of how dynamic the mRNA levels are.

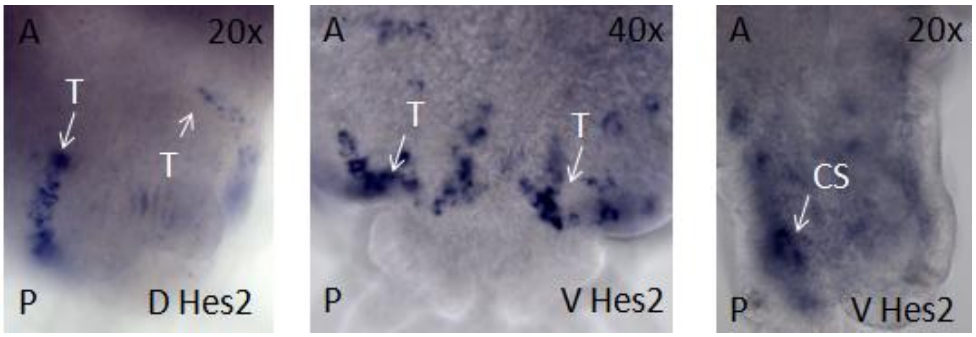

Fig. 19 - Dorsal and ventral location of Hes2 mRNA. (1) Incomplete ring of teloblasts (T); (2) Incomplete ring of teloblasts (T); (3) Chaetal Sacs (CS).

The three-dimensional reconstruction of stacks acquired at the confocal microscope LSM 710 Zeiss is a technique used to reveal how Cdx and Hes2 are expressed discontinuously.

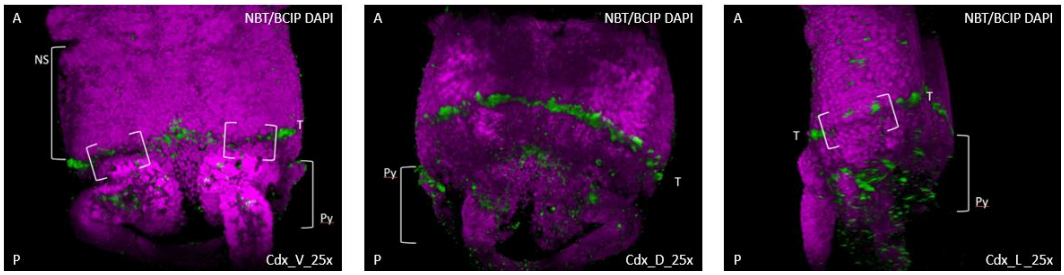

Fig. 20 - A three-dimensional reconstruction of the regenerate shows the location of Cdx in teloblasts. (1) The ventral view shows two main interruptions; (2) The dorsal view is showing a continuous expression of Cdx; (3) The left side clearly shows an interruption of signal. A = Anterior, P = Posterior.

The following confocal microscopy picture is showing a Cdx-labeled pygidium together with a clear ring of teloblasts.

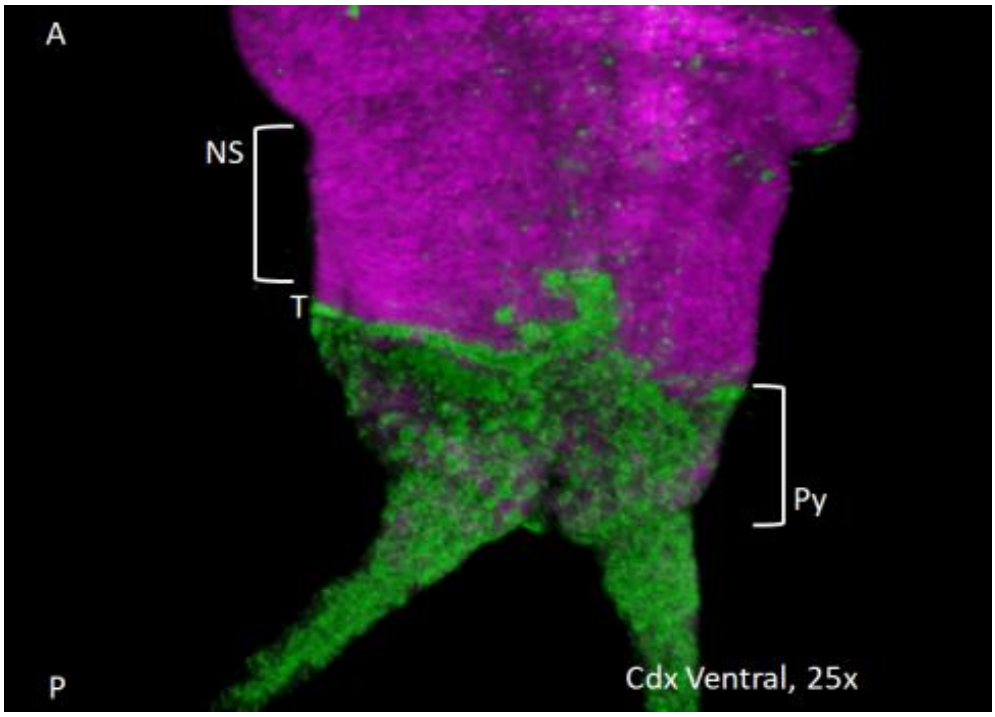

Fig. 21 - A three-dimensional reconstruction of a Cdx-labeled pygidium (Py). A ring of teloblasts is clearly identifiable (T). The new forming segments (NS) are unlabeled. A = Anterior, P = Posterior.

The following confocal microscopy pictures are showing the expression of Hes2 in teloblasts.

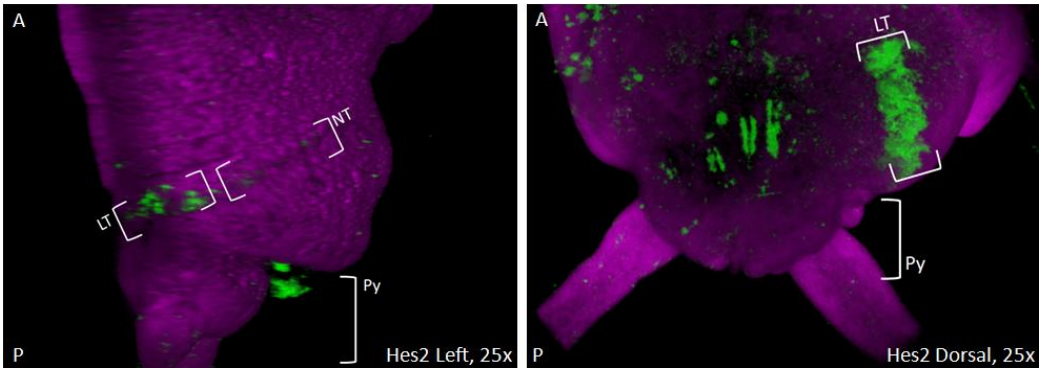

Fig. 22 - Two incomplete rings of teloblasts are labeled with Hes2. LT = Labeled teloblasts, NT = Non-Labeled teloblasts. The pygidium (Py) is not stained. The green particle next to the pygidium is background. A = Anterior, P = Posterior.

The expression of other Hes genes that are expressed in teloblasts was assessed too.

Total counts of labeled tissues

The following tables resume the localization of Cdx and Hes2 mRNAs. The stage of each regenerate is defined according to the classification proposed by Planques *et al*. [16]. After the stage 5, segment addition (seg.) starts. An abnormal regenerate is indicated by abn. The tables are clearly showing the oscillations of Cdx at the pygidium, in the gut and at the level of teloblasts and Hes 2 is oscillates only in teloblasts. From this in situ hybridization experiments it is not possible to reveal the full dynamics of Cdx and Hes2 cycle, being the numerosity of the samples too low for this purpose.

	Pygidium		Teloblasts		Gut	Hindgut	Neurons	Stripes	Stage
	Partial	Total	Partial	Total					
Cdx 1		Yes	Yes		No	Yes	Yes	No	2
Cdx 2	No	No		No	Yes	No	Yes	No	seg.
Cdx 3		Yes	Yes		Yes	Yes	Yes	No	seg.
Cdx 4		Yes	?	?	Yes	Yes	?	No	seg.
Cdx 5		Yes	?	?	Yes	Yes	No	No	abn.
Cdx 6		Yes	Yes		No	No	No	No	seg.
Cdx 8		Yes	Yes		Weak	Yes	No	Yes	2
Cdx 9	Yes		Yes		Yes	Yes	No	Yes	seg.
Cdx 10	Yes		Yes		Yes	Yes	No	Yes	seg.
Cdx 11		Yes	?	No	No	No	Yes	Yes	seg.
Cdx 12	No	No	?	No	Yes	No	No	Yes	seg.
Cdx 14		Yes	?	?	Yes	Yes	No	No	3
Cdx 15	No	No	No	No	Yes	Yes	No	No	4
Cdx 16	No	No	No	No	Yes	?	No	No	2
Cdx 18		Yes	?	?	Yes	Yes	No	No	seg.
Cdx 19		Yes	Yes		No	No	Yes	Yes	seg.
Cdx 20		Yes	Yes	?	Yes	Yes	No	Yes	5
Cdx 22		Yes	Yes		No	No	No	Yes	seg.
Cdx 24	Yes		Yes		No	Yes	Yes	No	5
Cdx 25	No	Yes		?	Yes	Yes	No	No	seg.
Cdx 26	No	No	No	?	No	Yes	No	No	seg.
Cdx 27	Yes		Yes		Yes	?	No	No	2
Total	4	12	11	0?	15	14	5	8	23

Fig. 23 - The localization of Cdx in 23 samples.

	Teloblasts		Chaetal sacs	Neurons	Stage
	Partial	Total			
Hes2 2	Yes		Yes	Yes	seg.
Hes2 5	Yes		Yes	No	seg.
Hes2 6	Yes		Yes	Yes	seg.
Hes2 7	Yes		Yes	Yes	seg.
Hes2 8	Yes		No	No	seg.
Hes2 9	Yes		?	Yes	5
Hes2 10	?	?	Yes	Yes	seg.
Hes2 11	No	No	Yes	Yes	3
Hes2 13	No	No	Yes	Yes	seg.
Hes2 14	No	No	Yes	Yes	seg.
Tot	**6**	**0**	**8**	**8**	**10**

Fig. 24 - The localization of Hes 2 in 10 samples.

To sum up my findings, I think that Cdx oscillates in a complex way. It must oscillate at the level of the pygidium, in teloblasts and in the gut. From these samples it is not clear whether the three cycles are somehow synchronized one another. At the level of teloblasts, I suspect a wave that starts ventrally and propagates to the dorsal side, like Prud'homme proposed for the Engrailed expression pattern [27].

One way to broaden the knowledge on Cdx and Hes2 transcriptional oscillation is to start to acquire data on one month juveniles. The *in situ* hybridization of juveniles can provide five hundred labeled individuals in a single experiment. I have already started to develop a proper protocol to this purpose, but the time constraint of the developmental time needed by the worms to reach the suitable stage made the experiments a little long. Moreover, the quality of the *in situ* hybridization for the genes Hes1, Hes4, Hes5, Hes6, Hes8 is for some reason rather poor. Possibly, the stock of the probes that were synthesized had some defects, hence I am planning to restart the whole experiment again.

Finally, I think that it is important to assess the cycling of these genes at a proteic level in teloblasts. This screening suggests that Cdx and Hes2 are likely to display oscillatory waves of expression. Therefore, I suggest producing their fluorescent reporter and to live image them as a prosecution of this study.

3.3 Involvement of Notch pathway in the molecular oscillator

Production of the transgene

The production of the transgene turned out to be harder than expected. The amplification of the 2-kb region by nested PCR in different individuals had many negative results. This suggests that the region of the primers is much more polymorphic than expected. Hence, I amplified the DNA from an inbred line, called FL2, whose genome is fully sequenced. The amplification was positive in all the samples. The transgene produced by Gibson Assembly is cloned in competent bacteria. The amplification of the plasmid that introduced mutagenesis on the PAM sites turned out to be tough too. In this case, I decided to remove one of the gRNAs from the mix, since I did not manage to mutate its PAM site. After a Gibson assembly, it is always important to sequence the region of a few colonies to make sure to have the exact sequence at the EGFP insertion point. Therefore, I verified that sequence of the transgene of a few clones by Sanger sequencing and selected the one that did not show any mutation compared to the expected sequence.

***In vitro* test of Cas9 activity**

The extracted plasmid is used to assess the functionality of the stock of Cas9 protein and of the duplexes trac-guide RNAs.

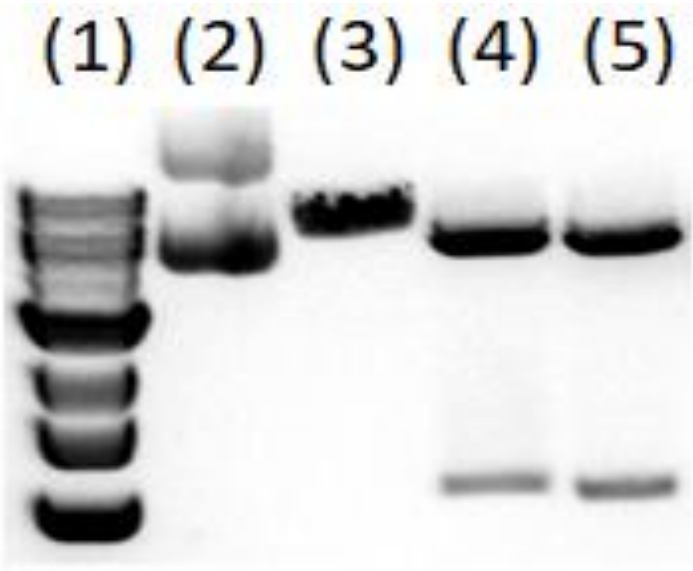

Fig. 25 - *In vitro* test of Cas9: Migration on a 1% agarose gel of: DNA ladder (1); the circular plasmid PCR2 containing the N-Cter-EGFP fragment (2); the linearised plasmid, obtained by *XbaI* digestion (3); the

previous plasmid, when subsequently digested by gRNA1 (4) and gRNA2 (5).

It is clear how the digestion by gRNAs of a linear molecule of DNA produces two distinct bands, therefore the complexes are perfectly functional.

In vivo test of Cas9 activity

To assess the functionality *in vivo* of the Cas9 complexes, we microinjected the Cas9 complex into fertilized eggs, as explained in the section materials and methods and let the larvae develop at 18°C for 24 hours. The DNA is recovered using the kit Phire Tissue Direct © and a PCR is performed to amplify a 200 bp region around the cut site. The genotyping turned out to be harder than expected, due to a contamination of the reagents that occurred at the beginning of my internship. A special PCR bench was dedicated to genotyping PCR and filtered tips were used all the times. The reagents and the water to dilute solutions have been changed all the time. Despite all the precautions that were used to avoid that, the contamination is still masking the possibly positive result. On the other hand, I cooperated with Florent Madouri, a master 1 student, whose aim was to fluorescently tag the gene Hox3 and to knock it in with a CRISPR/Cas9 protocol. Since the step of genotyping is relevant to the goal to develop a CRISPR/Cas9 protocol in *Platynereis*, I show that the obtained result is coherent with expectations.

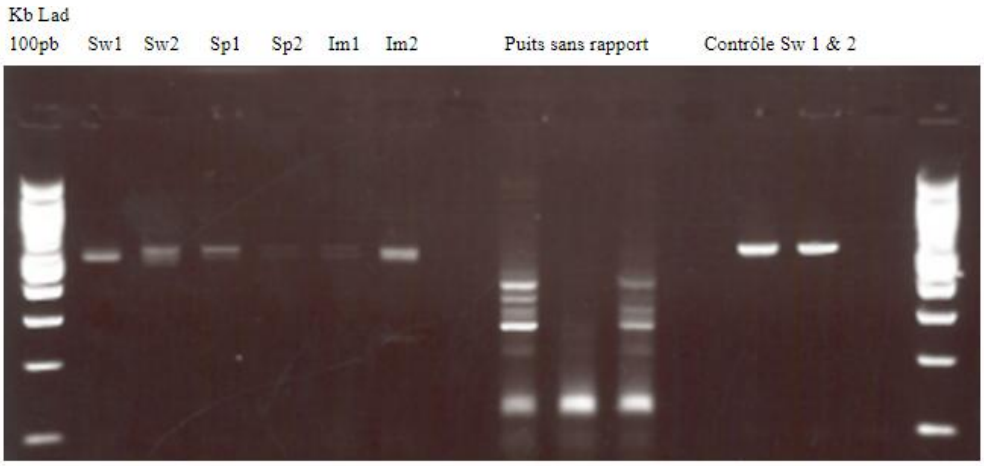

Fig. 26 - The injection of Cas9 without cassette produces different population of clones repaired by NHEJ. The six injected embryos are showing smears or multiple bands, while controls are displaying a single band, as we expected.

DNA sequencing at the cut site

Moreover, it is still important to sequence the amplified DNA. An optimal situation would make extremely clear the cut site. Indeed, when small insertions or deletions occur, we expect the downstream sequence frame to be totally altered, like in the picture. Again, I show the result obtained for the gene *Hox-3* together with Florent Madouri.

Fig. 27 - Sequence of the amplicon: small insertions/deletions produce different sequences downstream the cut site.

Controls

First of all, it is important to determine the overall survivability of the batch of fertilized eggs. Then, it is yet important to determine which percentage of mortality is due to the mechanical stress of the microinjection. It is possible to determine that, simply injecting the KCl Hepes buffer 1x as a control solution. Even in a low quality batch with the 40% of surviving individuals after 24 hpf, 78 injected eggs produced 31 normally developing embryos. Therefore, the mechanical stress is not particularly lethal for the embryo. To be sure that the signal is coming from a genomic context, it is important to determine that the single stranded DNA is not expressed. To do that, the single stranded DNA is injected without the Cas9 complexes. Since more than twenty animals are surviving in a pool of 70 injected embryos, I confirm that the concentration of ssDNA injected is not toxic.

Co-injection of the cassette + Cas9 and imaging of the transgenic lines

The fertilized eggs are injected and imaged as it is described in the section materials and methods. The picture below shows all the four swimming larvae 29 hpf that were obtained after the injection of Cas9 complexes 2 μM and ssDNA 5 ng/μL.

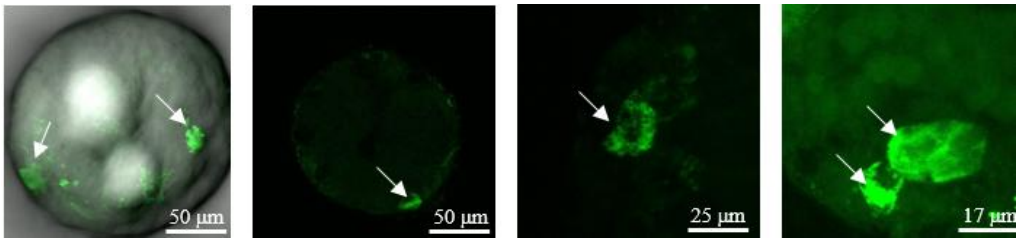

Fig. 28 - Four fluorescent abnormal embryos

It is clear that the embryos are not going to develop further, therefore the concentration of the Cas9 protein is still toxic. The strategy is to decrease more the concentration of the ribonucleoprotein to have greater percentages of surviving embryos. If the embryos are not surviving at lower concentration of ribonucleoprotein, the conception of the reporter should be revised. In this case it is possible to suspect that the insertion of EGFP at the carboxyterminal domain is hindering the functions of the receptor to some extent.

Once fluorescent mosaic individuals are obtained, as I already mentioned in the section materials and methods, it is important to prove that the fluorescent signal is coming from a labeled Notch receptor. To do this, I have already designed a couple of primers. The primer forward is located in the EGFP exogenous sequence, while the primer reverse is in the 3'UTR of Notch, out of the homology sequence used to do the Cas9 knock-in. This way, the DNA will be amplified only if the EGFP is integrated at the right site. Unfortunately, the limited duration of my stage and the problems encountered while I was building the chimerical DNA did not allow me to do this control yet.

Once the Notch receptor is successfully tagged in normally developing mosaic embryos, it is necessary to wait for them to mature about six months and to find at least one individual labeled in the germ line.

4. Conclusions and future directions

The genome editing protocol I started developing would be a milestone to fluorescently tag proteins in *Platynereis dumerilii*. It is now vital to continue injecting progressively lower concentration of ribonucleoproteic complexes to obtain living fluorescent worms. To gain more insights on the dynamics of the Cdx and Hes2 genes, it is now important to conduct the study on a numerous population of juveniles. Moreover, my work has already revealed that Cdx and Hes2 are two important genes that could participate in a segmentation oscillator in stem cells. They are candidate to be fluorescently tagged and provide a live oscillator reporter. Additionally, as I mentioned several times in my report, a number of other genes belonging to the Hes family are expressed in teloblasts and may be a relevant part of the posterior oscillator of *Platynereis* and I plan to assess their translation soon. Last, I am going to optimize the FUCCI tool that I tested to be used to assess the mitotic waves involved in segment addition. For example, the optimized FUCCI construct this can be used in combination with Wnt and Notch inhibitors to determine the role of these pathways in the regulation of the proliferation of stem cells.

Confocal microscopy was used on transgenic reporter worms to start to reveal the mechanisms underlying posterior addition of segments: those techniques may be considered sufficient to live image the worms for the issues that we have already investigated. However, a long-term perspective milestone would be to refine the imaging protocol both of larval development and juveniles' posterior elongation (the two main biological processes involving posterior addition), reaching a single cell resolution and a satisfying time resolution for the live movies. This involves the use of light sheet microscopy, and requires drug testing to anesthetize the worms without hindering posterior growth, triggering the necessity to develop microfluidic devices to deliver locally any kind of drug, without the need to interrupt the observation at the microscope. The refinement of all these protocols will eventually allow for the first time the observation of stem cells for the entire life cycle, using tools such as BRAINBOW to make a precise map of the teloblasts fate in segment addition.

Albeit there is still a lot of work to do, all the results together are laying the groundwork to a more ambitious goal. During the semester, I explored some of the genome editing and imaging techniques available for *Platynereis* and my personal feel is that it is the most suitable species to allow the live observation

and lineage tracing of highly multipotent stem cells in their undisturbed niche. It is broadly known that tumor masses are solely initiated, enhanced and propagated by a small fraction of cells displaying stem-like activity, that are very hard to image. Thus, I think that for this reason *Platynereis dumerilii* may also emerge as a model organism to study the involvement of the molecular niche on cell proliferation in cancer diseases.

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