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and

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APPENDAGE REGENERATION IN THE AMPHIPOD CRUSTACEAN *PARHYALE HAWAIENSIS*

ΑΝΑΓΕΝΝΗΣΗ ΤΩΝ ΑΚΡΩΝ ΣΤΟ ΑΜΦΙΠΟΔΟ ΚΑΡΚΙΝΟΕΙΔΕΣ *PARHYALE HAWAIENSIS*

PhD thesis

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under the supervision of Dr. Michalis Averof

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Abstract

Regeneration is the restoration of a body part after injury or as a normal bodily process. Many animals are capable of regenerating different body parts. Salamanders regenerate their tail and legs, crabs regenerate their appendages, flatworms can regenerate their entire body, humans can regenerate part of their liver, etc. The phylogenetic distribution of regenerative capacity in the animals is still unresolved. There are closely related species with very different regenerative capacities. To understand how regeneration is achieved and how regenerative capacity evolved in the Metazoa, we need to collect information from diverse species.

Traditionally studied model organisms, such as flies, *C. elegans* and mice have poor regenerative capacities. On the other hand, animals with extended regenerative capabilities, such as starfish, crabs, flatworms and salamanders, are not amenable to genetic manipulation. *Parhyale hawaiensis* is capable of regenerating all of its appendages within approximately one week after amputation. Moreover, work from our and other labs over the last ten years has developed a series of tools, rendering *Parhyale* an attractive model for regeneration studies.

The main goal of my PhD thesis was to establish *Parhyale hawaiensis* as the first arthropod regeneration model organism. To achieve this, I described limb regeneration in *Parhyale*, I studied the level of commitment of progenitor cells that generate the new tissue and identified a cell type that participates in muscle regeneration in Parhyale. I compared my findings with what is known in other regeneration models to gain information about the evolutionary history of tissue regeneration.

Initially, I identified that *Parhyale* is able to regenerate all of its appendages within 5-8 days, restoring all of the appendages' cell types, such as epidermis, neurons and muscles. I established a timeline of the process of appendage regeneration in *Parhyale*, identifying five distinct stages. (a) Wound closure, (b) blastema formation, (c) patterning, (d) growth and (e) muscle regeneration.

Animals that are capable of regeneration employ two very different strategies. Planarians use totipotent cells to regenerate their missing parts. On the other hand, vertebrates activate committed progenitors to regenerate specific tissues. To examine the level of commitment of the progenitors during *Parhyale* limb regeneration, I generated mosaic animals expressing a fluorescent protein under the control of the heat-shock promoter in specific lineages. I subjected these animals to amputation and assessed the participation of the labeled cells in regeneration. I identified that the progenitors are lineally restricted and reside locally, indicating the absence of a single pool of progenitors. These experiments clearly indicate that *Parhyale* resembles vertebrates in that it uses committed rather than totipotent progenitors.

I also identified a Pax3/7-expressing mesodermal cell type closely attached to the muscle fibers. These cells were highly reminiscent of the muscle satellite cells that have been described in vertebrates; therefore, I named them satellite-like cells (SLCs). Homologs of satellite cells had never been identified earlier outside the chordates.

Satellite cells have been described in vertebrates to participate in muscle repair, growth and homeostasis. They are molecularly characterized by the expression of the Pax3/7 family of transcription factors. Their participation in limb, fin and tail regeneration differs among species. By Edu staining and transplantation experiments, I was able to show that SLCs participate in the formation of the blastema, as well as in muscle formation during appendage regeneration.

Collectively, these results highlight a number of key similarities in regeneration between arthropods and vertebrates, arguing for common cellular mechanisms that were present in their last common ancestor. Moreover, the discovery of SLCs in arthropods pushes back the origin of satellite cells to the last common ancestor of protostomes and deuterostomes, at the base of the bilaterians. This could imply that the ability to regenerate muscles has a common origin dating back to precambrian times. Alternatively, muscle regeneration may have evolved independently in arthropods and vertebrates, engaging a homologous cell type – satellite cells – as the source of regenerated muscles.

Περίληψη

Η αναγέννηση αποτελεί μια φυσική διαδικασία για την αντιμετώπιση εξωτερικών προκλήσεων που απειλούν τη σωματική ακεραιότητα του οργανισμού. Πολλοί οργανισμοί αναγεννούν διαφορετικά μέρη του σώματός τους. Οι σαλαμάνδρες αναγεννούν την ουρά και τα πόδια τους, τα καβούρια αναγεννούν τα άκρα τους, οι πλατυέλμινθες μπορούν να αναγεννήσουν οποιοδήποτε μέρος του σώματός τους, οι άνθρωποι αναγεννούν τμήμα του ήπατος κοκ. Η αναγεννητική ικανότητα ποικίλλει ανάμεσα σε διαφορετικούς οργανισμούς. Υπάρχουν πολύ κοντινά είδη ζώων τα οποία διαφέρουν σημαντικά στην αναγεννητική τους ικανότητα. Για να καταλάβουμε πώς επιτυγχάνεται η αναγέννηση και πώς εξελίχθηκε η αναγεννητική ικανότητα στα Μετάζωα, πρέπει να συλλέξουμε πληροφορίες από διαφορετικά είδη.

Τα "κλασσικά" γενετικά συστήματα, όπως η Δροσόφιλα, ο νηματώδης C. elegans και το ποντίκι, παρουσιάζουν πολύ μικρή αναγεννητική ικανότητα. Αντίθετα, σε οργανισμούς στους οποίους παρατηρείται σημαντική αναγεννητική ικανότητα, όπως στους αστερίες, στους πλατυέλμινθες, στα καρκινοειδή και στις σαλαμάνδρες, δεν διαθέτουμε ακόμα ανεπτυγμένα γενετικά εργαλεία για τη μελέτη της αναγέννησης. Τα τελευταία χρόνια ένα νέο πρότυπο γενετικό σύστημα – το καρκινοειδές *Parhyale hawaiensis* – έχει αναπτυχθεί, προσφέροντας νέες δυνατότητες για μελέτη του φαινομένου της αναγέννησης. Ο *Parhyale* παρουσιάζει πλήρη αναγέννηση των άκρων εντός μίας εβδομάδας από την απόσπασή τους.

Κύριος στόχος της διδακτορικής μου διατριβής ήταν η καθιέρωση του αμφιπόδου καρκινοειδούς *Parhyale hawaiensis* ως μοντέλου για τη μελέτη της αναγέννησης. Στα πλαίσια της διδακτορικής μου διατριβής περιέγραψα τη διαδικασία της αναγέννησης στον *Parhyale*. Στη συνέχεια, μελετήσα την προέλευση και την πλαστικότητα των προγονικών κυττάρων που συμμετέχουν στην παραγωγή του νέου ιστού. Ακολούθως, μελέτησα την αναγέννηση των μυών, εντοπίζοντας έναν κυτταρικό τύπο που συμμετέχει στην αναγέννηση των μυών στον *Parhyale*.

Αρχικά, έδειξα ότι ο *Parhyale* μπορεί και αναγεννά όλα τα άκρα του μέσα σε 5-8 μέρες, αποκαθιστώντας όλους τους κυτταρικούς τύπους, όπως επιδερμίδα, νευρώνες

και μύες. Διέκρινα πέντε στάδια στην αναγέννηση άκρων στον *Parhyale*: (α) Επούλωση της πληγής, (β) σχηματισμός βλαστήματος, (γ) μορφογένεση, (δ) αύξηση, (ε) αναγέννηση μυών.

Διαφορετικά ζώα χρησιμοποιούν διαφορετικές στρατηγικές για να αναγεννήσουν μέρη του σώματός τους. Οι πλατυέλμινθες χρησιμοποιούν ολοδύναμα κύτταρα, ενώ τα σπονδυλωτά χρησιμοποιούν διαφοροποιημένα προγονικά κύτταρα που παραμένουν δεσμευμένα στην αρχική τους ταυτότητα. Πραγματοποίησα ανάλυση γενεαλογιών με χρήση μεταθετών στοιχείων και ανακάλυψα ότι τα προγονικά κύτταρα του *Parhyale* παραμένουν δεσμευμένα στην αρχική τους γενεαλογία και βρίσκονται κοντά στον αναγεννώμενο ιστό. Η αναγέννηση, λοιπόν, στον *Parhyale* ομοιάζει σε αυτή των σπονδυλωτών και όχι των πλατυέλμινθων, όσον αφορά την πλαστικότητα των προγονικών κυττάρων.

Ανακαλυψα επίσης ένα μεσοδερμικό τύπο κυττάρων που εκφράζει το μοριακό δείκτη Pax3/7 στενά συνδεδεμένο με τις μυικές ίνες. Αυτά τα κύτταρα μοιάζουν σημαντικά με τα μυικά δορυφορικά κύτταρα των σπονδυλωτών, γι'αυτό τα ονόμασα satellite-like cells. Κύτταρα ομόλογα των μυικών δορυφορικών κυττάρων δεν έιχαν βρεθεί ξανά σε μη χορδωτά.

Τα δορυφορικά κύτταρα στα σπονδυλωτά συμμετέχουν στην αύξηση, επιδιόρθωση και ομοιόσταση των μυών. Η συμμετοχή τους στην αναγέννηση διαφέρει ανάμεσα στα είδη. Με χρώση πολλαπλασιαζόμενων κυττάρων και πειράματα μεταμόσχευσης, έδειξα ότι αυτά τα κύτταρα στον *Parhyale* συμμετέχουν στο σχηματισμό του βλαστήματος καθώς και στο σχηματισμό των μυών κατά την αναγέννηση.

Τα αποτελέσματα της διατριβής μου καταδεικνύουν σημαντικές ομοιότητες στη διαδικασία της αναγέννησης στα αρθρόποδα και τα σπονδυλωτά, προτείνοντας κοινούς κυτταρικούς μηχανισμούς παρόντες στον τελευταίο κοινό τους πρόγονο. Επίσης, η ανακάλυψη μυικών δορυφορικών κυττάρων στα αρθρόποδα υποδεικνύει την πιθανή ύπαρξη μυικών δορυφορικών κυττάρων στον τελευταίο κοινό πρόγονο πρωτοστομίων και δευτεροστομίων. Αυτό θα μπορούσε να σημαίνει ότι η ικανότητα αναγέννησης μυών υπήρχε πριν την Κάμβρια περίοδο. Εναλλακτικά, η αναγέννηση των μυών μπορεί να εξελίχθηκε ανεξάρτητα στα δύο φύλα, χρησιμοποιώντας έναν ομόλογο κυτταρικό τύπο, τα μυικά δορυφορικά κύτταρα, ως πηγή των αναγεννημένων μυων.

Chapter 0

Prologue

The two greatest discoveries in Modern Biology are Darwin's description of evolution accompanied by the identification of natural selection as its driving force and the discovery of the nature of the genetic material together with its structure that supports its role. These were, in my opinion, the most recent *paradigm shifts* in biological research and have led to a revolution in evolutionary and molecular biology. These discoveries were made in the middle of the 19th and the 20th century, respectively. Several discoveries after these can be considered equivalent. Sequencing of the human genome, the discovery of genetic regulatory regions and their role in development, cell cycle regulation and cell death, the identification of mobile genetic elements or small RNAs, the production of induced pluripotent stem cells from differentiated cells and other significant discoveries have shaped biological research over the last century. Recent advancements in informatics, technology and biology may be breeding the third revolution, the integrative revolution.

The beginning of this century has been marked by the development of tools that provide access to an ample landscape of data. Whole genome sequencing techniques, microarrays, RNA-Seq, ChIP-Seq, transcriptomics, interactomics, proteomics etc are techniques that have arisen the last years and flooded research with data. It is now matter of time and money to accumulate terabytes of sequences that represent different stages of development, environmental conditions, age, time-points etc, whatever one can imagine. The question reasonably follows. What do all these data mean? How do we extract interesting, significant information out of this ocean of data? How do we draw conclusions?

When designing a line of research, the way one should think is: (a) What scientific question is of my interest? What do I want to learn? (b) How will I generate the data I need in order to answer this question? How are these data going to help me approach this question? (c) Once I have these data, how do I extract the information I need in order to answer this question? As the collection of data is facilitated by new technologies, the interest in scientific research is being shifted towards the scientific questions and the way to take advantage of the available data.

The extraction of the relevant information looks like searching a needle in a haystack; hence, the development of new algorithms that can fulfill this need using the least amount of time and space is very challenging. This area of informatics research is of great interest and will be so for the next years.

On the other hand, the scientific questions we can approach are much less restricted than in the past, since we are now able to gain access to this amount of information. We can nowadays appreciate the complexity of nature and acquire an integrative answer to our queries. By integrative, I mean a solution that would integrate information from different sciences, different disciplines or different experimental contexts. This is going to be especially advantageous for the field of Evolutionary Biology. The capability to integrate more and different types of data while comparing two or more taxa is critical for drawing more robust conclusions and acquiring a better view to their history. Moreover, the assessment of the accumulated information requires the integration of diverse sciences into biological research, such as informatics and physics.

Nowadays, we do not really need to restrict our analyses in a small number of species that offer very specific advantages. In this direction, evolutionary developmental biology has started to successfully expand its questions beyond the classical model organisms. However, apart from comparing developmental processes, the existence of diverse model species will also allow us to compare other non-developmental processes, such as wound healing or tissue maintenance and regeneration, and conclude on their ancestral relationships.

Parhyale is an amphipod crustacean that has been established as a genetic model in our lab over the last ten years. My thesis diverged from the evolutionary developmental approach, since I became interested in the capacity of this creature to very efficiently regenerate its appendages. My goal was to study the cellular basis of *Parhyale* appendage regeneration, in order to be able to approach two general questions: a) what is the plasticity of regeneration progenitors in different animals and b) how has regenerative capacity evolved within the Metazoa?

In Chapter 1, I describe *Parhyale* as a model system and some tools that were designed or adapted during my thesis. In Chapter 2, I describe some basic aspects of *Parhyale* appendage regeneration, which are necessary in order to put the rest of my work in the appropriate context and I approach the first question regarding the nature of regeneration progenitors that participate in appendage regeneration. In Chapter 3, a putative progenitor population is identified and evaluated for its regenerative capacity. Finally, in the last Chapter I make an effort to compose the available knowledge and envisage the future of regeneration research.

Chapter 1

Parhyale hawaiensis

Understanding biological processes requires the acquisition of information from a wide variety of species. However, our knowledge is restricted to data originating from a non-random subset of species from the tree of life. The animals that have been selected for biological studies have to fulfill certain prerequisites in order to serve as model systems. The animals from which we have obtained lots of information are the ones that have a short life cycle, generate many progeny, are easy to breed in the lab and for which enough background knowledge exists. This generates a cycle, in which we generalize ideas that are true only for a few species and reinforce these ideas by testing them in the same animals. This has led to the situation where most of the labs across the world work with worms, flies, mice and, lately, zebrafish.

In order to overcome this limitation, it is important for biologists to invest in establishing new models that span the tree of life. This will offer the opportunity to answer biological questions in diverse contexts. People will also be able to compare the accumulated knowledge from traditional model organisms with what is observed in other organisms and generate knowledge concerning the evolution of specific traits. To this end, significant progress has been made in the field of evolutionary developmental biology, where tools have been developed in representative species from different phyla.

The development of genetic tools is currently the bottleneck for the utilization of different species as biological models. In order to be able to perform functional studies, we need to be able to access the genetic material of the organism of choice and to manipulate it. Moreover, whichever manipulation is achieved is important to be able to be transmitted to the next generation. Therefore, the biggest effort nowadays is put into generating genetic tools. As technology evolves, new genetic tools become increasingly complex and difficult to be implemented in one's favorite organism. However, there are certain inventions, which can be applied universally and have proven to be very helpful for developmental biologists.

In this first chapter of my thesis, I will describe my organism of choice, *Parhyale hawaiensis,* an arthropod crustacean, which has emerged the last ten years as the first crustacean genetic model with a variety of different tools in our arsenal. After describing the animal, the available tools and its significance as a model system, I will focus on my contribution to the development of tools and techniques that are suitable for *Parhyale*.

Introduction

1.1 Parhyale hawaiensis

Parhyale has a typical arthropod body plan, consisting of multiple segments, appendage-bearing or not. The anterior part of the body forms the cephalic region, which carries the first antennae, the second antennae, the mandibles, the first maxillae and the second maxillae, successively. The first thoracic appendage, which is also used for feeding, is very closely attached to the cephalic region and carries the maxilipeds. The next four segments of the thoracic region (T2-T5) bear appendages, which face anteriorly, whereas thoracic segments T6-T8 carry appendages, which face posteriorly, hence the name of the Order Amphipoda (from the greek words αμφι, which means "both" and πόδι meaning "leg"). The thoracic appendages differ from each other depending on their use. The second and third thoracic appendages (T2-T3, gnathopods) are involved in grabbing and mating. Especially, the third appendage is sexually dimorphic, being evidently larger in males than females. The remaining thoracic appendages (T4-T8, pereopods) are mainly involved in locomotion. The abdominal region consists of the last six segments and contains two different types of appendages. The first abdominal segments (A1-A3) bear the pleopods, which function as swimming aids. The last three segments (A4-A6) carry the uropods (Figure 1) (Browne, Price et al. 2005).

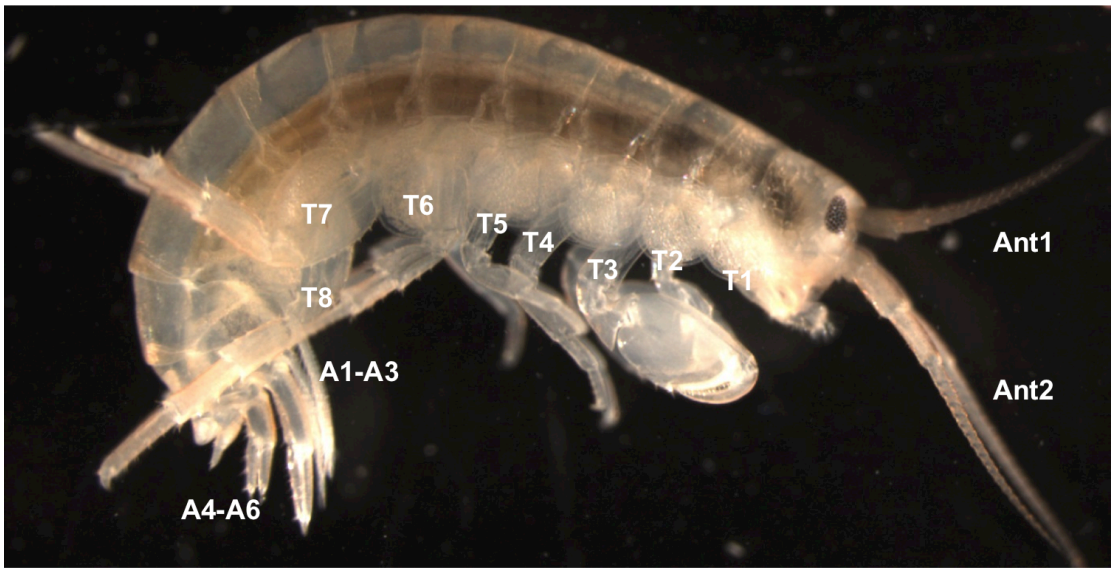


Figure 1: ***Parhyale hawaiensis***
Parhyale hawaiensis body plan and appendages. Ant1-2: antennae, T1-8: thoracic appendages, A1-6: abdominal appendages.

Parhyale's generation time is approximately 2 months at 26^{O}C. The male grabs the female before mating and they form a couple until the female oviposits, when the male fertilizes the eggs. After fertilization, the female carries the embryos in a pouch situated ventrally between the thoracic appendages during embryogenesis, which lasts 10-12 days. After embryogenesis, the animal hatches, it grows for approximately seven weeks, until it is sexually mature and ready to generate progeny.

1.2 Embryogenesis

Embryogenesis in *Parhyale* has a total duration of ten to twelve days at 26^{O}C. *Parhyale* embryos are easily accessible and can be extracted from the pouch of the female. They are fairly transparent during all stages of embryogenesis, which has been described in detail (Browne, Price et al. 2005).

Parhyale has a stereotypic early cell lineage. The first two cleavages of embryogenesis are slightly asymmetric, whereas the third one is highly asymmetric. This results in an eight-cell stage embryo that consists of four large blastomeres, termed macromeres, and four small ones, the micromeres. It has been shown that each one of the blastomeres gives rise to a specific cell lineage. The macromeres give rise to the left ectoderm (El), right ectoderm (Er), the posterior ectoderm (Ep) and the anterior and visceral mesoderm (Mav). On the other hand, the micromeres will be the

ancestors of the right mesoderm (mr), the left mesoderm (ml), the endoderm (en) and the germline (g) (Gerberding, Browne et al. 2002) (Figure 2).

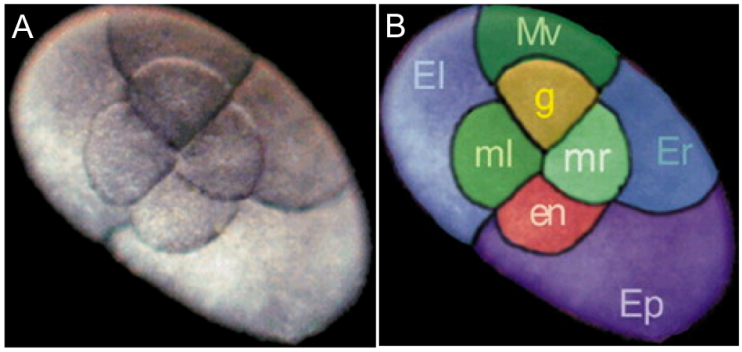


Figure 2: *Parhyale*'s determinate early cell lineage
In the 8-cell stage embryo of *Parhyale*, each blastomere has a distinct identity, with a stereotypic cell lineage and germ-layer contributions. El: left ectoderm, Er: right ectoderm, Ep: posterior ectoderm, Mv: anterior and visceral mesoderm, ml: left mesoderm, mr: right mesoderm, en: endoderm, g: germline. Figure taken from (Gerberding, Browne et al. 2002).

The transparency of the embryos and the facility of manipulation have enabled studies of *Parhyale* early embryogenesis. Although the blastomere lineages are stereotypic at the eight-cell stage embryo, after ablation of one of the mesodermal or ectodermal blastomeres, intra-germ layer compensation is observed until gastrulation but not later. However, inter-germ layer compensation never takes place between ectodermal and mesodermal lineages (Price, Modrell et al. 2010). Therefore, if all three of the germ layer's blastomeres are ablated, the animal does not hatch. It has also been observed that ectoderm can generate segments in the absence of mesoderm but not vice versa (Hannibal, Price et al. 2012). Moreover, the cell divisions and movements until gastrulation have been thoroughly described, providing a very useful framework for future studies (Alwes, Hinchen et al. 2011) (Chaw and Patel 2012).

1.3 Genetic tools

During the last decade there has been a concerted effort from several labs to establish genetic tools in order to make *Parhyale* an attractive genetic model. Collectively, a number of different genetic, genomic and imaging tools have been developed, as summarized in Table 1.

Genetic tools	
Transposable element driven transgenesis	Pavlopoulos and Averof, 2005
Site-directed integration (attP/attB)	Kontarakis et al, 2011
Gene mis-expression	Pavlopoulos et al, 2009
Gene knockdown	Liubicich et al, 2009 Ozhan-Kizil et al, 2009
Genetic mosaic analysis	**in this study, in progress**
Gene trapping and trap conversion	Kontarakis et al, 2011
Inducible cell ablation	Ioanna Koltsaki, master thesis
Genomic resources	
ESTs	JGI project (genome.jgi-psf.org/parha/parha.home.html)
Transcriptomes	Zeng et al, 2011 Blythe et al, 2012 Zeng et al, 2012
BACs	Parchem et al, 2010
Imaging tools	
4D microscopy	**in progress**
OPT/SPIM	**in this study, in progress**

Table 1: Genetic, genomic and imaging *Parhyale* toolkit

In order to perform functional genetics in *Parhyale*, it is indispensable to be able to genetically transform the animals. The Minos transposable element (Franz and Savakis 1991) can be used to achieve stable transformation of *Parhyale* (Pavlopoulos

and Averof 2005). The transposable element carrying the desired insert and a transformation marker can be injected in the zygote alongside with the Minos transposase, and the G1 animals are screened for stable transformants. If one injects the transposable element in the 2-cell stage embryo, a mosaic animal is created where only the left or the right half of the animal carries the transgene. This can prove to be very useful in case one wants to use the wild-type half of the animal as a negative control of the transformed half.

A gene-trapping approach has also been established in *Parhyale* with significant efficiency (Pavlopoulos and Averof 2005), which has helped us generate a variety of gene-trap lines (Kontarakis, Pavlopoulos et al. 2011). Recently, we have also developed a versatile tool for generating and exploiting gene traps in *Parhyale* (iTraC – integrase mediated Trap Conversion), which can be potentially adapted to other non-model organisms (Kontarakis, Pavlopoulos et al. 2011). iTraC offers the advantage of converting gene traps that are manifested by fluorescent protein expression into one's favorite genetic tool. By inserting an attB site in the trapping construct, we were able to convert the expression of DsRed in the distal parts of the appendages of a pre-existing gene trap line (that carries an attP landing site) into EGFP. Using iTraC, one is able to express any desired protein in the pattern of the trapped gene. (Figure 3)

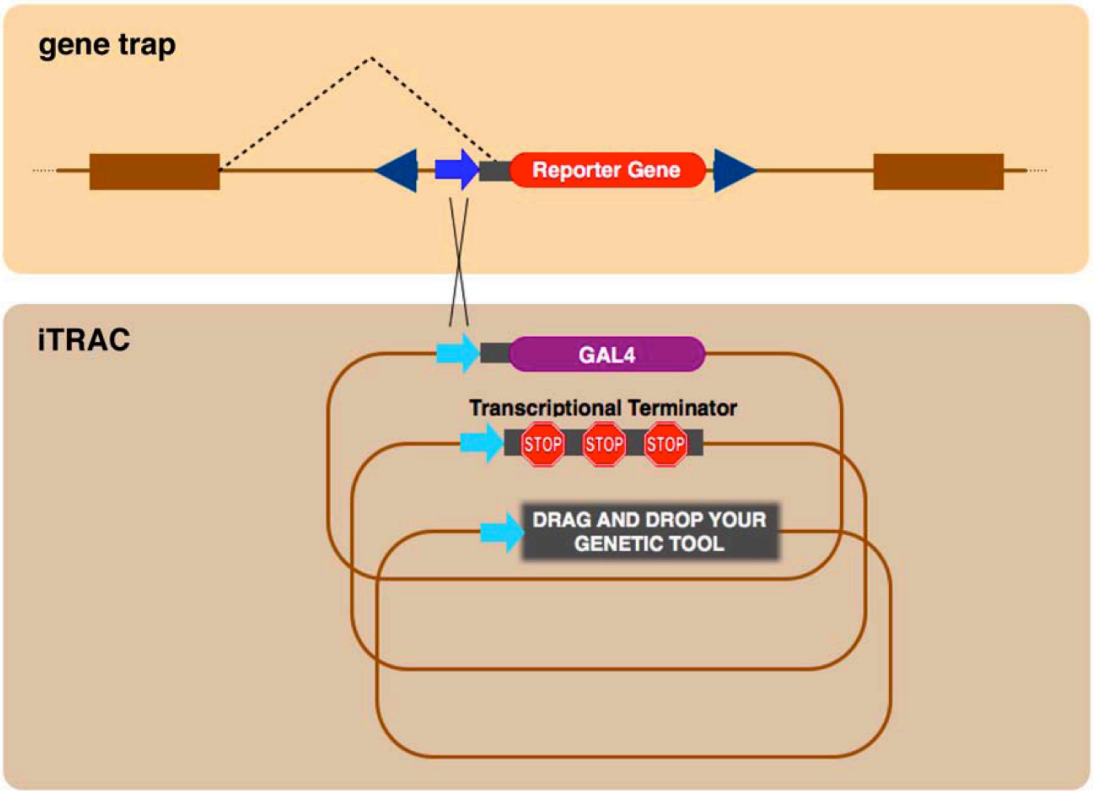


Figure 3: iTRAC
iTRAC (integrase-mediated Trap Conversion) allows the exploitation of a specific gene trap for the expression of any desired genetic tool in the pattern of the trapped gene. Figure taken from (Kontarakis, Konstantinides et al. 2011).

Apart from the aforementioned techniques, it is also possible to overexpress genes in *Parhyale* by using a heat-shock inducible promoter, which is activated ubiquitously (Pavlopoulos, Kontarakis et al. 2009), and to knock genes down using RNAi (Liubicich, Serano et al. 2009) or morpholinos (Ozhan-Kizil, Havemann et al. 2009). Moreover, we have introduced an inducible cell ablation technique, which was originally developed in zebrafish (Curado, Anderson et al. 2007; Curado, Stainier et al. 2008; Koltsaki 2012).

Although *Parhyale's* genome is not sequenced yet, a number of different transcriptome datasets have become available during the last years. With an overall goal of providing the necessary genomic resources for comparative and developmental studies, the *Parhyale* community has sequenced and assembled transcriptomes coming from ovaries (maternal contribution of transcripts in the embryo) and embryos (zygotic transcription) (Zeng, Villanueva et al. 2011; Blythe, Malla et al. 2012). Moreover, *Parhyale* small RNAs have been sequenced identifying

this way both conserved and arthropod-specific miRNAs (Blythe, Malla et al. 2012). Finally, a BAC library that covers five times the genome of *Parhyale* has been constructed and has been successfully screened for several developmentally relevant genes (Parchem, Poulin et al. 2010).

1.4 Functional studies

Besides generating tools, a number of functional studies have been performed underlining the importance of research in non-model species by revealing information that would be inaccessible by studying only the traditional models. I will briefly mention a few examples to show how some of the previously mentioned tools were put into use.

The role of Ubx in the specification of thoracic appendages was first implied fifteen years ago (Averof and Patel 1997), but the appropriate tools for directly testing this hypothesis were not available. After the development of misexpression and knock-down tools in *Parhyale*, the role of Ubx was rigorously assessed and it has been confirmed that the anterior retraction of Ubx expression has been, indeed, the driving force for the evolution of maxillipeds (Liubicich, Serano et al. 2009; Pavlopoulos, Kontarakis et al. 2009).

Spliced-leader trans-splicing is performed by the addition of a common spliced-leader sequence, which carries a modified 5' cap, upstream of a transcript. The function and the evolution of this process are still largely unknown. Through analysis of *Parhyale* EST datasets, it was estimated that 10% of *Parhyale* transcripts are spliced-leader transpliced. The expansion of this survey in other species showed that the phylogenetic distribution of spliced-leader transplicing is sparse, letting the researchers propose multiple gains of this ability (Douris, Telford et al. 2010).

Crustaceans are a sister group to insects. Therefore, *Parhyale* can serve as an ideal organism for comparing processes studied in *Drosophila* and drawing conclusions about their evolution. As an example, by interfering with the expression of *single-minded*, which specifies the ventral midline in *Drosophila,* it was found that this gene has the same function in *Parhyale,* arguing for the presence of a *single-minded* specified midline in the common ancestor of insects and crustaceans (Vargas-Vila, Hannibal et al. 2010). Conversely, it was found that Vasa expression, which is

necessary for germ line formation in *Drosophila,* is not essential for the formation of the germ line in *Parhyale* but only for its maintenance, raising a discussion concerning the evolution of Vasa function in the germ line (Ozhan-Kizil, Havemann et al. 2009).

Results - Discussion

The genetic arsenal that was described in the introductory part of this Chapter is necessary for founding a model species, but in the course of my thesis it proved to be insufficient. In order to answer the functional questions that will be described in the Chapters that follow, I often found myself in need of new tools. The invention and/or adaptation of new tools in different organisms can be time-consuming and involves a great deal of trial and error. In order to spare researchers the trouble of repeating experiments already performed, I will present here a variety of tools and techniques I tested, most of which were not successful.

1.5 The quest for a promoter with ubiquitous activity

The ability to constitutively and ubiquitously express a protein of interest is necessary for several applications. Although the *Parhyale* heat-shock promoter (*Ph*HS) (Pavlopoulos, Kontarakis et al. 2009) drives ubiquitous expression, it is only activated as a response to high temperature. A promoter with ubiquitous activity was initially necessary for me for the lineage tracing experiments (see Chapter 2) and, also, for the establishment of the Cre/lox recombination system that will be described in the following section.

EF1a promoter

The first fragment I tested for potential ubiquitous promoter activity was the EF1a upstream region. This is a genomic region of about 5.7 kilobases that includes the transcription start of the *Parhyale* EF1a (Elongation Factor 1a), which was kindly provided by EJ Rehm and NH Patel. This promoter had been described to be strongly active during embryogenesis (Rehm EJ and Patel NH, unpublished).

In order to test its functionality in postembryonic stages, I cloned the regulatory region upstream of a fluorescent protein (DsRed) and inserted it in the Minos construct. I injected the construct alongside with Minos transposase mRNA in zygotes and followed its activity by the expression of the fluorescent protein in 21 transgenic embryos. The promoter was, indeed, active until the end of embryogenesis, although with decreasing intensity. Once the animals hatched, they stopped expressing the fluorescent protein (Figure 4). Since I was interested in driving transgene expression

during adulthood and, specifically, during appendage regeneration, this particular promoter proved to be of no use.

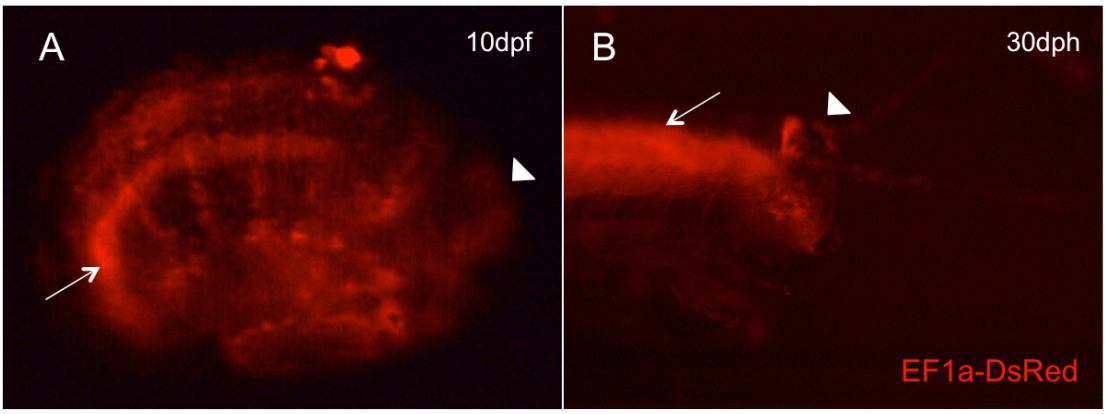


Figure 4: EF1a promoter-driven DsRed expression
(A) EF1a promoter drives the expression of DsRed ubiquitously until the end of embryogenesis. (B) In transgenic adults, the expression is significantly reduced, or absent. In this particular animal, some residual expression can be seen in the brain (arrowhead). dpf: days post-fertilization, dph: days post-hatching. Both the embryo and the adult are oriented with the head facing to the right (arrowheads). The arrows denote the autofluorescence of the gut.

Spliced-Leader (SL) and U1 snRNA promoters

One of the problems in exploring *Parhyale*'s genome for regulatory regions is the big intergenic regions and the capability of regulatory elements to exert their function over large genomic distances. A good place to seek a ubiquitous promoter would be a genomic region densely occupied by genes that are constitutively expressed. One candidate is the genomic region where the trans-splicing spliced-leader sequences are located.

10% of the genes of *Parhyale* are estimated to be trans-spliced (Douris, Telford et al. 2010). Trans-splicing, just like cis-splicing, seems to be a ubiquitous process, since there have not been reports of cell-specific expression of spliced leader (SL) sequences. In *Parhyale*, SL sequences are organized in 1.4 kb and 2.4 kb repeats. In the 1.4 kb repeat, between the two SL sequences there exists a U1 snRNA gene, which should also be consitutively expressed, since it is a component of the spliceosome.

I decided, together with Ismene Karakasilioti, to test for ubiquitous promoter efficiency (a) the region that lies between U1 snRNA and the second SL sequence in both directions – one might drive the transcription of the U1 snRNA and the other the

transcription of the SL sequence - (named SL_U1 and U1_SL, respectively) and (b) the genomic region between the two SL sequences that might guide the transcription of the third SL sequence (termed SL_SL). Each of the three genomic regions was placed upstream of DsRed and in the Minos vector and injected in 1-cell stage *Parhyale* embryos. The SL_SL driver did not show any activity at any stage of embryogenesis or in early hatchlings. U1_SL and SL_U1 drove the expression of DsRed in restricted areas. U1_SL was active, but not in all cells, until stage S18 (staging after (Browne, Price et al. 2005)). DsRed expression was not detected afterwards. SL_U1 was active more broadly than U1_SL, and the expression persisted until the end of embryogenesis, although with decreasing intensity. However, after hatching the expression, if any, was undetectable (Karakasilioti 2009). In conclusion, as with EF1a, the result was also disappointing.

White Spot Syndrome Virus (WSSV) immediate early promoters

A practice commonly employed for the identification of ubiquitous promoters is the utilization of promoters from viruses that are infectious in the species of interest. For example, a cytomegalovirus (CMV) immediate early gene promoter is widely used for constitutive expression in mammals (Boshart, Weber et al. 1985). The same is true for a simian virus 40 (SV40) early gene promoter (Byrne, Davis et al. 1983). Viruses' early promoters rely on the transcription machinery of the host. Viral immediate early genes are the first ones to be activated after the infection of the host and are expressed without the aid of other viral proteins. Consequently, they are very good candidates when looking for ubiquitous promoters.

The White Spot Syndrome Virus (WSSV) is a virus infecting crustacean populations with high mortality rate (Lu, Wang et al. 2005). It affects shrimps, crabs and crayfish (van Hulten, Witteveldt et al. 2001). Three immediate early genes, *ie1, ie2* and *ie3* have been identified in the WSSV genome. The first two immediate early genes are activated within two hours after infection, *ie1* being more intense. The activity of *ie1* promoter was also assayed in *Sf9* insect cell cultures (Liu, Chang et al. 2005) and primary shrimp cells (Lu, Wang et al. 2005) where it drives efficiently the expression of EGFP.

I amplified the *ie1* and *ie2* immediate early gene promoters from WSSV genomic DNA that was provided by Professor Just Vlak. The WSSV genomic DNA was

prepared from isolates collected from naturally infected *Penaeus monodon* from Thailand in 1996, and it has been sequenced (van Hulten, Witteveldt et al. 2001). After amplification, I subcloned the regulatory regions upstream of DsRed, inside the Minos vector and injected the constructs in *Parhyale* 1-cell stage embryos as was done with the previous candidate promoters. Both of the immediate early gene promoters, *ie1* and *ie2,* were found to be inactive in *Parhyale* embryos and adults.

1.6 Recombination techniques

One of the most widely used genetic tools for lineage tracing and genetic manipulation is site-specific recombination. Site-specific recombination is achieved through the irreversible recombination of two recognition sites that is driven by the activity of a recombinase. Once this alteration is achieved, the cells that had expressed the recombinase are irreversibely marked and can be traced or manipulated. Usually, the way these techniques are employed is by flanking a strong transcriptional terminator with the recombination sites and putting this combination upstream of a marker (eg. a fluorescent protein) or a functional protein (eg killer protein). Once the recombination occurs, the transcriptional terminator is eliminated and the downstream gene is transcribed. This happens exclusively in cells that have expressed the recombinase.

Cre/lox recombination

Cre ("Causes recombination") recombinase is a bacterial protein that recognizes specific (lox) sites and recombines them without the need for cofactors.

To prove that Cre is active in *Parhyale*, before creating transgenic animals, I performed a plasmid-based assay (similar to assays used for testing trasposase activity (Klinakis, Loukeris et al. 2000)) in *Parhyale* embryos. I injected 50 embryos (1-, 2- and 4-cell stage) with a plasmid carrying the lox sites flanking a 550 bp region (pPax(LoxN-HindIII fragment-LoxN) – see Appendix B) together with capped mRNA of the Cre recombinase. After 24 hours, I performed a Holmes-Bonner plasmid extraction, and performed a PCR on the extracted plasmid. Depending on the activity of the recombinase, I would get a 280 bp band (active Cre) or a 830 bp band (inactive Cre). As demonstrated in Figure 5, Cre is active in *Parhyale* embryos.

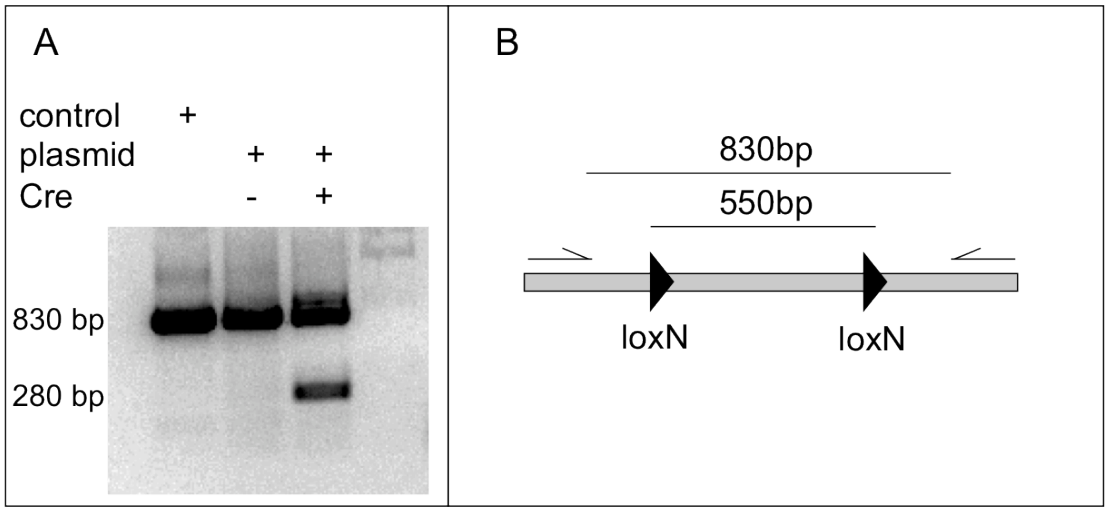


Figure 5: Testing the functionality of Cre/lox using an in vivo excision assay
(A) Gel showing the results of the Cre/lox excision assay. The pPax(LoxN-H3-LoxN) plasmid was injected into 1-cell stage embryos with or without Cre mRNA. After 24 hours, the plasmid was recovered by Holmes-Bonner plasmid extraction and PCR was performed on the extract. Cre activity causes the excision of a 550bp fragment from the plasmid, generating an extra PCR band. In the first lane, the control is a PCR performed on uninjected plasmid. The next two lanes carry PCR products from injections with or without Cre mRNA. (B) The pPax(LoxN-H3-LoxN) plasmid is illustrated alongside with the primers that were used for the excision assay.

In order to apply the Cre/lox system for a detailed cell lineage analysis, I decided to use the Brainbow technology (Livet, Weissman et al. 2007). The Brainbow construct 1.1M, which I used, consists of four different fluorescent proteins which are separated by different lox variants – loxN, lox2272, loxP (Livet, Weissman et al. 2007). These variants are recognized by Cre and can be recombined, but only identical lox sites recombine with each other. The outcome is that in each cell where recombination occurs one of the fluorescent proteins is going to be expressed stochastically. Depending on the specific time point of Cre activation, one can trace the descendants of several cells carrying independent recombination events, within the same animal. In order to avoid background expression of OFP (Orange Fluorescent Protein - Kusabira), I used a version of Brainbow 1.1M that carries a stop codon in the Kusabira ORF, made by Johannes Schinko.

The ideal way of performing the lineage tracing experiments would be to have a ubiquitous promoter upstream of the Brainbow construct and a Cre recombinase under an inducible (heat-inducible in our case) promoter. Transgenic lines carrying each of these constructs would then be crossed to bring the two transgenes in the same animal. To test the Brainbow system in the absence of a ubiquitous promoter, I used

the heat-inducible promoter in both constructs. I tested one line for each construct. I crossed stable transformants from each line with each other and brought both Cre and Brainbow in the same embryos, confirmed by the presence of both markers (3xP3.DsRed in the case of Brainbow and 3xP3.EGFP in the case of Cre recombinase). I screened 5 embryos for the activity of the Brainbow system by heat-shocking in different embryonic stages (at the fourth day of embryogenesis and at the end of embryogenesis); I was not able to detect any fluorescent protein expression.

There are many reasons that could explain why this approach has been unsuccessful, as for example the toxicity that has been associated with Cre recombinase in proliferating cells in mice as well as in *Drosophila* (Schmidt, Taylor et al. 2000; Heidmann and Lehner 2001; Loonstra, Vooijs et al. 2001). Moreover, I could have checked more lines for each transgene, as the inefficiency could be attributed to position effects, or more embryos from the existing line. Given that Brainbow was, at the time, thought to be ineffective in other organisms, I decided not to pursue the investigation further and try to adapt the Flp/FRT recombination system instead.

Flp/FRT recombination

Flp is also a recombinase, similar to Cre. The two main differences is the recognition sites, which are called FRT sites for Flp, and the fact that it was isolated from a eukaryotic organism, specifically, from *Saccharomyces cerevisiae* (Cox 1983).

Similarly to what I did with Cre, I performed a plasmid-based assay to test whether Flp is active in early *Parhyale* embryos. I injected 60 embryos (1-, 2- and 4-cell stage) with a mix consisting of a plasmid with FRT sites flanking a 800 bp region (pMi(FRT-FRT) – see Appendix B) and capped mRNA of Flp recombinase. After 24 hours, I extracted the plasmids and performed a PCR on the extract. Depending on the activity of Flp, I expected a 400 bp band (active Flp) or a 1.2 kb band (inactive Flp). Figure 6 clearly indicates that Flp is active in early embryos. Both Cre and Flp excision assays were performed in collaboration with Ioanna Koltsaki.

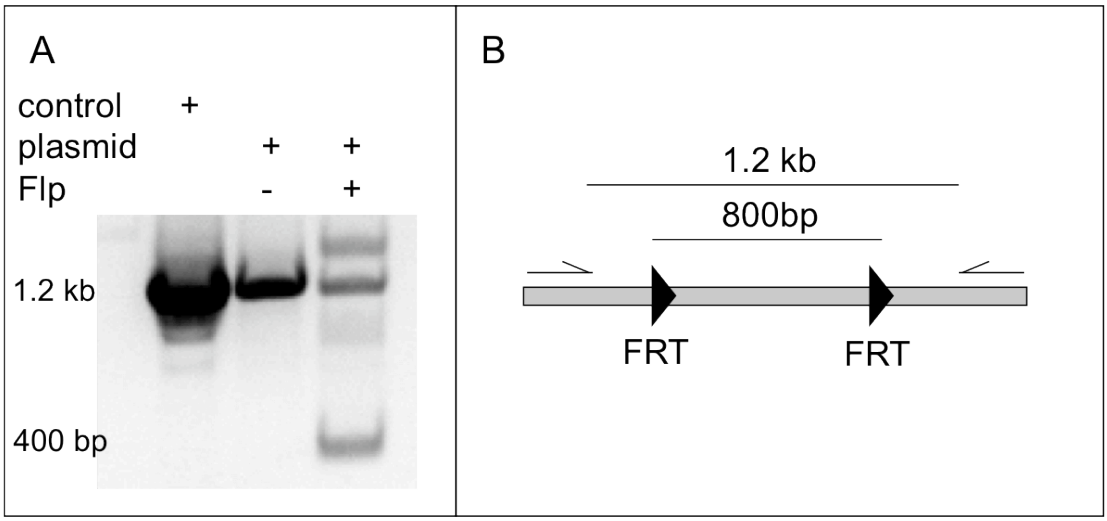


Figure 6: Testing the functionality of Flp/FRT using an in vivo excision assay
(A) Gel showing the results of the Flp/FRT excision assay. The pMi(FRT-FRT) plasmid was injected into 1-cell stage embryos with or without Flp mRNA. After 24 hours, the plasmid was recovered by Holmes-Bonner plasmid extraction and PCR was performed on the extract. Flp activity causes the excision of a 800bp fragment from the plasmid, generating an extra PCR band. In the first lane, the control is a PCR performed on uninjected plasmid. The next two lanes carry PCR products from injections with or without Flp mRNA. (B) The pMi(FRT-FRT) plasmid is illustrated alongside with the primers that were used for the excision assay.

Subsequently, I decided to generate two transgenic lines, one carrying the Flp under the *Parhyale* heat-inducible promoter (*Ph*HS) and another carrying a construct that bears a non-fluorescent protein (*Drosophila* lamin) followed by two *Drosophila* polyadenylation signals (*hsp70Aa* and *hsp27*) between the FRT sites and a fluorescent protein downstream. The construct was modified from the Flybow 2.0 construct provided by Iris Salecker (Hadjieconomou, Rotkopf et al. 2011).

In order to avoid recurring activation of Flp after every heat-shock, I decided to add another layer of control by using a tamoxifen-inducible form of Flp. In this scheme, Flp is merged to a mutated mammalian estrogen receptor ligand binding domain (LBD) which, when unbound, prevents the hybrid protein from entering the nucleus (Leone, Genoud et al. 2003) and, consequently, Flp cannot exert its function. Hydroxytamoxifen is an estrogen receptor antagonist, which binds the mutated estrogen receptor LBD with high affinity and allows it to enter, alongside with Flp, in the nucleus. The estrogen receptor LBD in this hybrid has been mutated to decrease the affinity to estrogen and increase the affinity to hydroxytamoxifen (Metzger, Clifford et al. 1995; Feil, Brocard et al. 1996). Therefore, Flp, which is called FlpER in this context, is inactive, in the absence of hydroxytamoxifen. I constructed two

plasmids carrying two different versions of FlpER that were provided by Gerhart Ryffel (Werdien, Peiler et al. 2001) under the control of the heat-shock promoter. (a) FlpER(T) carries two mutations in the estrogen receptor LBD, V400G and G521R, whereas (b) FlpER(T2) has four mutations, V400G, G521R, M543A and L544A.

Transgenic lines carrying the inducible forms of Flp, FlpER(T) and FlpER(T2), have been generated, whereas the transgene carrying the FRT sites has been injected many times, but I have not managed to recover any transgenic lines. A plausible reason for this might be toxicity associated with leaky expression of the *Drosophila* lamin carried in this construct.

1.7 Cell-specific markers

The existence of markers for specific cell types, time-points, environmental conditions etc would be really helpful to study the behavior of different cell populations during biological processes, such as regeneration. The trapping element pMi(3xP3-DsRed;*Ph*Hsp70a-DsRed) has been used to generate gene traps that express DsRed in specific cell types (Kontarakis, Pavlopoulos et al. 2011). To enrich our collection of traps, we decided to perform a gene-trap screen in our lab.

To facilitate the production of gene traps and circumvent the need for microinjections, we decided to create a transgenic line that would be the source of Minos transposase. The objective was to combine a transgenic line bearing the Minos transposase gene under control of the heat-shock promoter stably inserted into the genome, and a second transgenic line, carrying the Minos element. The latter was already existent in the lab. We created the construct shown in Figure 7A. The experimental procedure for creating a stable transgenic line that carries the Minos transposase was the one shown in Figure 7B. Unfortunately, after the induction of Minos transposase expression with heat-shock, no traps were observed in more than 50 heat-shocked embryos or in the progeny of 7 treated animals. This could be due to a piRNA-mediated response in these animals. Therefore, we decided to proceed with the gene-trap screen the way it was originally published (Kontarakis, Pavlopoulos et al. 2011).

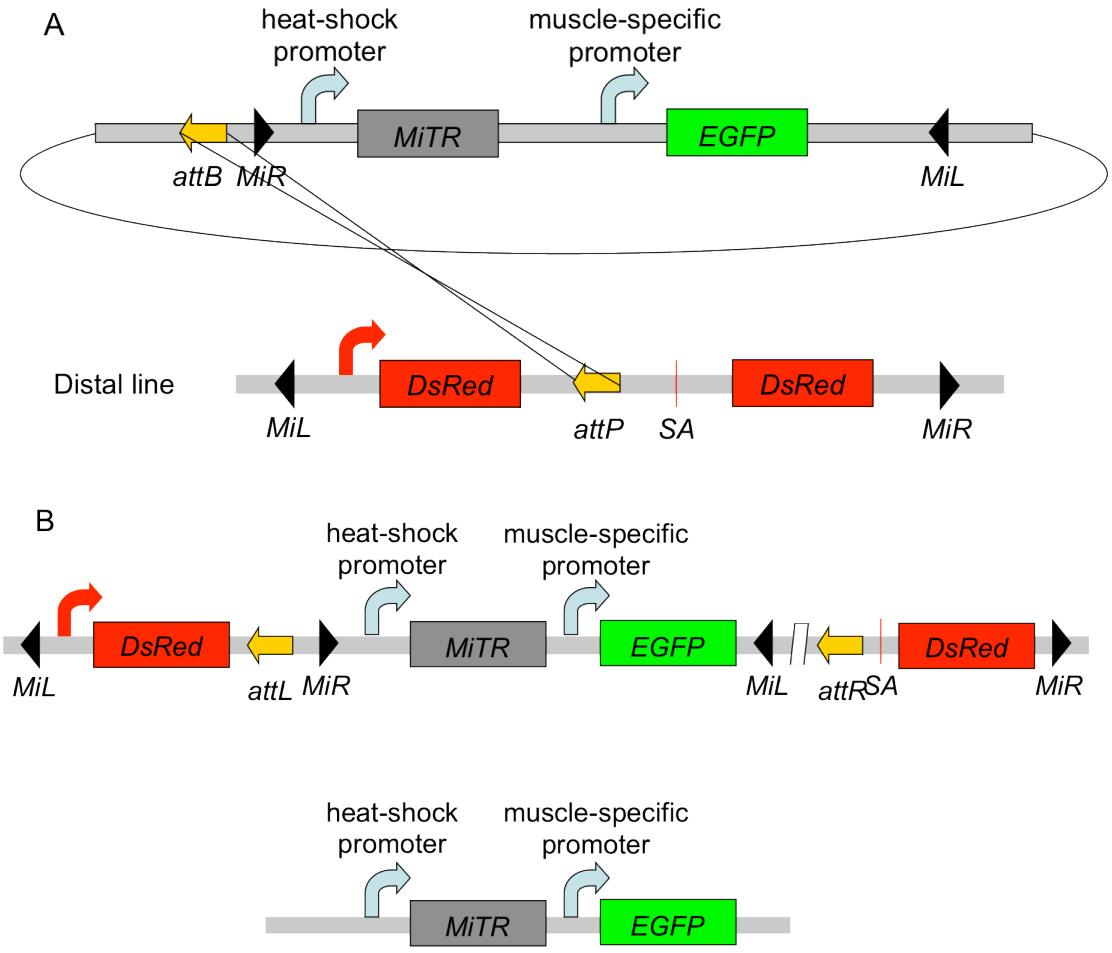


Figure 7: Generation of a Minos transposase-expressing transgenic line
(A) In order to generate a Minos transposase-expressing transgenic line that would not carry mobiliseable Minos transposable elements, we constructed a plasmid carrying an attB site (pBS(attB;MiR;*Ph*HS-MiTR;*Ph*MS-EGFP;MiL), upper part) and injected it in embryos of the *Distal* gene-trap line that have attP sites integrated in their genome (lower part). (B) After integration of the plasmid, the line carries Minos transposase under the control of a heat-shock promoter associated and PhMS-driven EGFP, as a marker. (C) By inducing transposase expression by heat-shock we would be able to excise the fragments flanked by Minos inverted repeats upstream and downstream of the transposase, generating a line carrying stably integrated Minos transposase.

Apart from the gene-trap screen, which is a long-term project, I decided to scrutinize the already existent reporter lines for useful expression. During my Master thesis, I had injected a construct that contained DsRed under the control of a multimerized DC5 enhancer-promoter element. DC5 is recognized by a Pax6/Sox2 transcription factor heterodimer in mice (Kamachi, Uchikawa et al. 2001), whose binding results in transcription initiation. Moreover, its function appears to be conserved between the chick and the fly (Blanco, Girard et al. 2005). Using the DC5-DsRed reporter line, I had observed the expression of DsRed in the central nervous system, during embryogenesis and adulthood. When I looked at its expression in the appendages in

adults, I noticed that DC5 was also driving DsRed expression in a nerve that was traversing the whole appendage (Figure 8). This is the only available neural marker in *Parhyale* appendages so far.

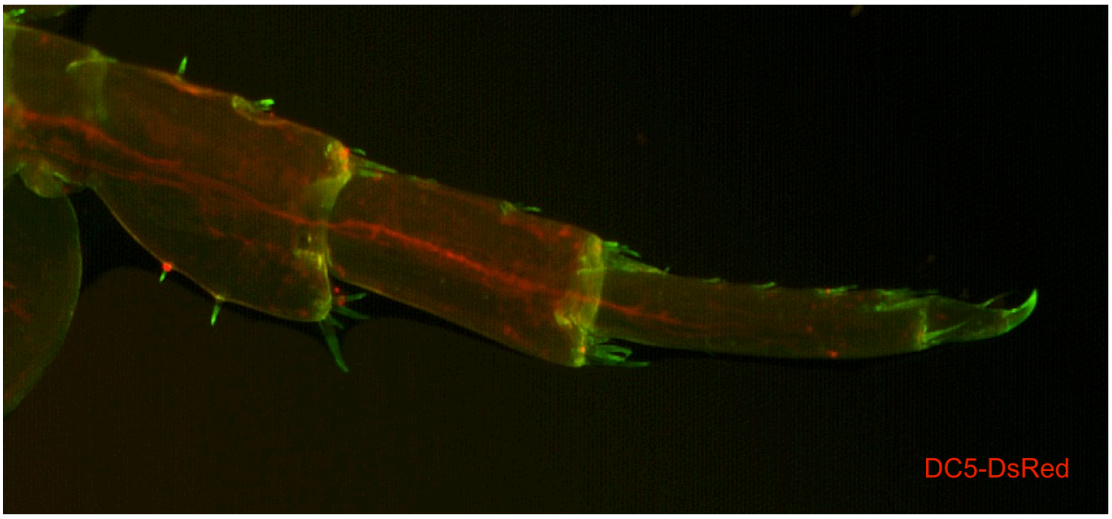


Figure 8: DC5-driven DsRed expression in appendage nerves
The DC5 element (Blanco, Girard et al. 2005) drives the expression of DsRed in nerves that are traversing all the appendages. The autofluorescence of the cuticle can be seen in green.

1.8 Imaging (OPT-4D)

Optical imaging is a rapidly advancing technological field. Developments in microscopes, instruments, data storage, image analysis and correction algorithms have made the field flourish over the last decade. Different imaging technologies have been developed and are already widely applied in biomedical research, such as Fluorescence Molecular Tomography (Ntziachristos, Schellenberger et al. 2004), Optical Projection Tomography (Sharpe, Ahlgren et al. 2002), Selective Plane Illumination Microscopy (Huisken, Swoger et al. 2004), multiphoton microscopy, etc. We wanted to see whether these techniques could also be applicable in *Parhyale*, who has the imaging advantage of having a relatively transparent exoskeleton.

OPT

Optical Projection Tomography (OPT) is a noninvasive imaging technique, which can be combined with fluorescence labeling (Sharpe, Ahlgren et al. 2002). The development of image analysis algorithms facilitates the three-dimensional reconstruction of images taken from the same sample in different angles, giving a

high quality insight on the 3D structure of the specimen. Moreover, it is very efficient with specimens in the range of tens of micrometers up to about a centimeter (small animals or organs of bigger animals). It can also allow for live imaging.

With the help of a post-doc from Jorge Ripoll's lab, Udo Birk, we used the setup depicted in Figure 9.

In order to employ OPT in *Parhyale*, we imaged animals of the *Ph*MS-DsRed transgenic line that express DsRed in the muscles (Pavlopoulos and Averof 2005). We needed to find the best way to stabilize the animal during the imaging procedure. Initially, we placed *Parhyale* in the glass capillary, as shown in Figure 10A. We also tried gluing the animal at the end of the capillary (Figure 10B). However, the reconstructions with the latter setup were poorer in quality, since it was difficult to restrict the glue, which was interfering with imaging.

The specimens were incubated in 40mM Natrium azide for 1h prior to the experiment, and transferred to a viscous mounting medium consisting of 4% methyl-cellulose in sea water. Afterwards, they were mounted in the capillary and imaged with the OPT setup. A reconstruction from one of these experiments is shown in Figure 11. (Birk, Rieckher et al. 2010; Birk, Darrell et al. 2011).

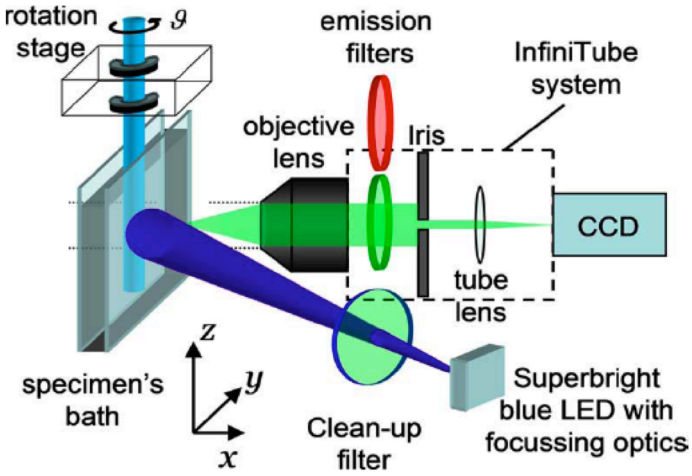


Figure 9: OPT setup
The specimen is mounted in a capillary and placed in a seawater bath. The capillary is attached onto and controlled by a rotation stage. The specimen is illuminated either with white light or excited by a super bright LED and the image is captured by a CCD camera. Diagram taken from (Birk, Darrell et al. 2011).

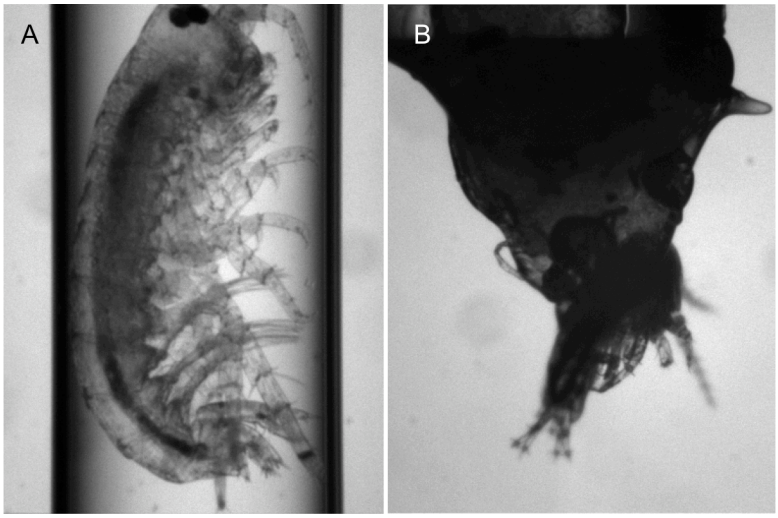


Figure 10: Mounting of *Parhyale* in a capillary for imaging by OPT
Two different ways of mounting *Parhyale* into a capillary were tried. (A) The aminal can be imaged by mounting *Parhyale* into a glass capillary. However the difference in the refractive indices of the seawater and the glass of the capillary made the reconstructions more difficult. (B) Gluing *Parhyale* underneath the capillary. As is evident from the figure, the density of the glue interfered significantly with the imaging of the animal.

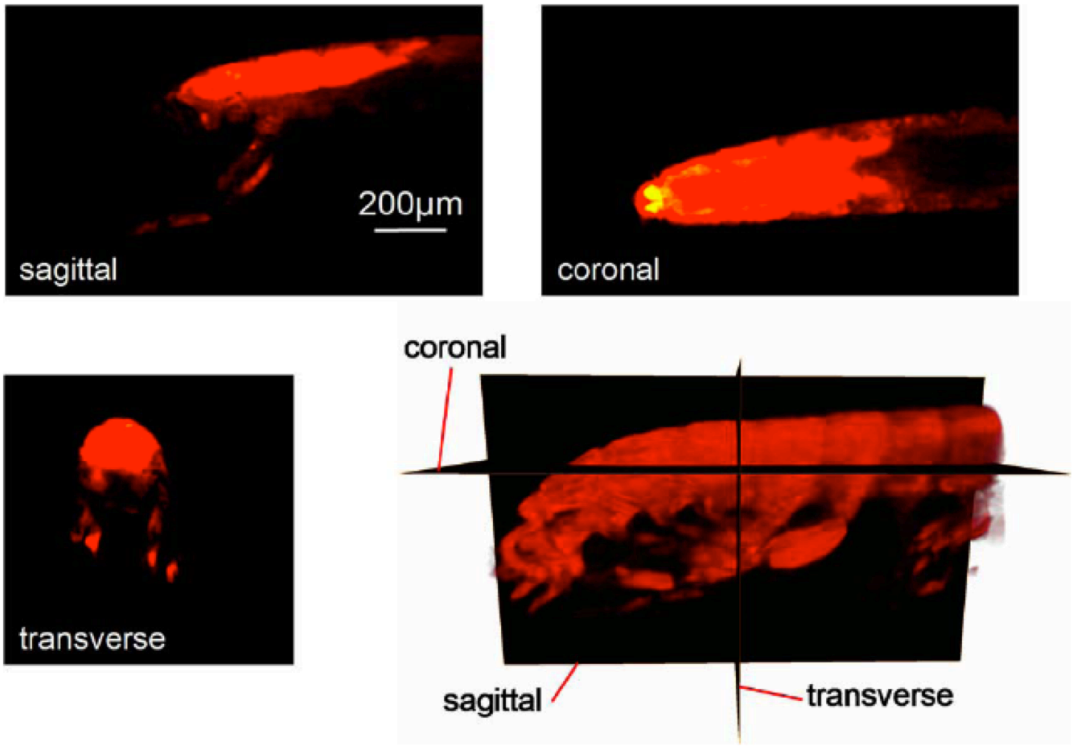


Figure 11: Imaging a *Ph*MS-DsRed animal with the OPT setup
Sagittal, coronal and transverse sections from the 3D reconstruction of the images obtained with the OPT setup are shown. Image taken from (Birk, Darrell et al. 2011).

SPIM

Selective Plane Illumination Microscopy is a technique that accomplishes optical sectioning eliminating background fluorescence from the planes that are out of focus. This is realized by scanning the excitation laser to create a single, thin, light sheet and arranging the objective lens perpendicular to the sheet (Figure 12). Fluorescent molecules that lie outside the illumination plane will not be excited (i.e. will not fluoresce) and, therefore, will not be recorded by the objective. By scanning the specimen in multiple parallel planes and in a handful of angles, one can reconstruct a three-dimensional image of the specimen.

By mounting *Parhyale* hatchlings of the Distal trap line (Kontarakis, Pavlopoulos et al. 2011) exactly as described for the OPT setup, we were able to acquire the reconstructions shown in Figure 13.

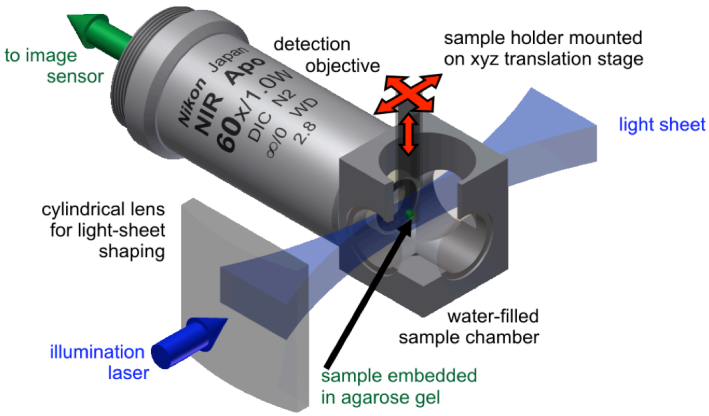


Figure 12: Single Plane Illumination Microscopy
SPIM has the advantage that the laser illuminates only a thin slice (single plane) of the sample. The plane of illumination is perpendicular to the objective lens, eliminating out-of-focus fluorescence. Diagram taken from
http://www.dkfz.de/Macromol/research/pic/spim_principle_large.png

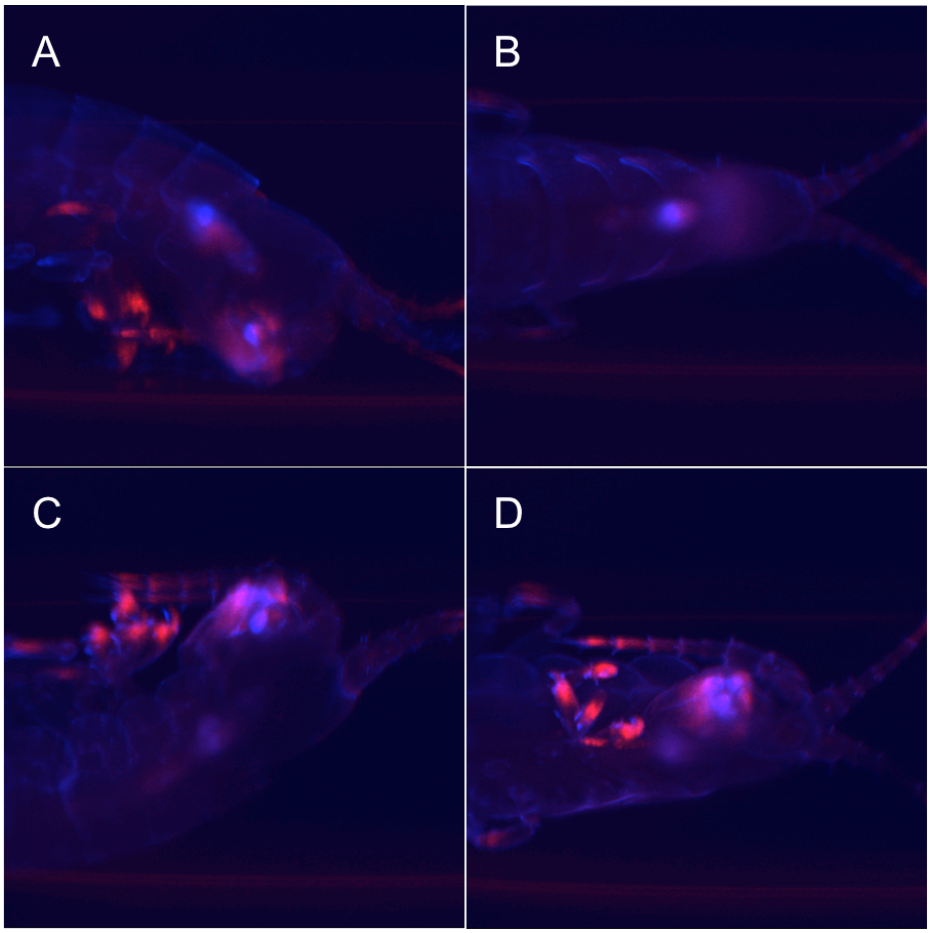


Figure 13: Imaging a Distal-DsRed animal with the SPIM setup
Different angles of an animal from the Distal line obtained with the multiview SPIM setup available in the Ripoll lab, at IESL-FoRTH.

Low magnification live imaging

Some biological processes do not need to be recorded at high magnification. For example, if one needs to image a whole appendage, the aforementioned techniques are useless. The main issue in order to perform live imaging is immobilizing the animal for long periods (a day up to a week) and keeping the tissue to be imaged completely still. I managed to achieve that by gluing the imaged appendage with surgical glue on a cover slip and mounting it with plastelin in a big Petri dish, as shown in Figure 14. The animal can be fed and remains alive and kicking until molting when it is released from the glue.

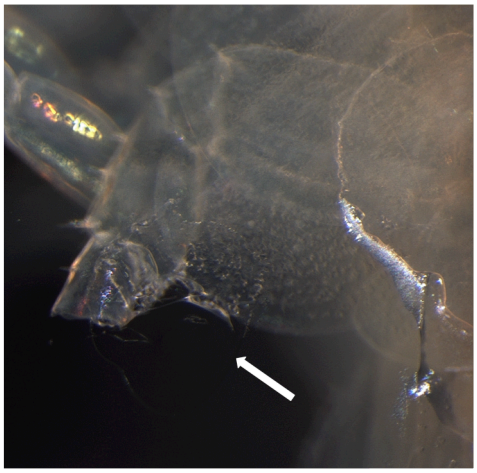


Figure 14: Low magnification live imaging of *Parhyale* limb regeneration
It is possible to image a living animal by gluing it on a cover slip, immobilizing the limb that is being imaged, and by supporting the coverslip (inverted) using plasteline spacers in a large petri dish. The appendage is firmly glued on the cover slip (white arrow) while the animal is alive and active.

Chapter 2

Parhyale hawaiensis as a regeneration model

Regeneration is the renewal, regrowth, or restoration of a body part or tissue after injury or as a normal bodily process. Within this definition is incorporated a broad spectrum of different phenomena that are triggered by diverse stimuli and operate under different mechanisms. Practically every organism has the capability to regenerate in this sense. Obviously, the extent of regenerative capacity varies significantly between different organisms. The phylogenetic distribution of the extent of the regenerative capacity in the animals is still unresolved. Therefore, regeneration research is very appealing for evolutionary biologists.

Flatworms can regenerate almost every body part starting from a small body region. Several arthropods can regenerate their appendages. Salamanders are also capable of regenerating many different tissues. However, if one marks in the tree of life the species that can regenerate extensively and the species that do not, it is clear that there is no consensus in the way regenerative capacity has evolved over time. There are even extreme examples where species within the same genus differ in their regenerative capacity; *Lineus ruber* and *Lineus viridis* are two worms that are hard to tell apart, but one of them (*L. ruber*) has capacity for bidirectional regeneration, whereas the other (*L. viridis*) has no regenerative ability (Brockes and Kumar 2002). Studying regeneration in detail in different organisms could facilitate our understanding of the evolution of regeneration.

Understanding regeneration will also help us appreciate the similarities and differences this process shares with embryonic development. It is still an open question whether the process of limb regeneration, for instance, is equivalent to embryonic limb development and can be, therefore, considered as "adult development". It is not known whether the regenerating structure utilizes the same genetic program as its embryonic counterpart or whether it has evolved different mechanisms to accomplish full restoration.

Until relatively recently, there was an established idea in biology that once cells became committed to a certain lineage, there was no way of changing their fate. Since most of the developmental pathways are characterized by a gradual restriction in developmental potential, it was widely believed that these pathways were unidirectional. This is, indeed, the case under normal developmental conditions. However, this "dogma" started to be challenged fifteen years ago (Bjornson, Rietze et al. 1999; Clarke, Johansson et al. 2000; Toma, Akhavan et al. 2001). Research in regeneration contributed significantly in this area, offering specific examples where the "dogma" was disputed (Echeverri and Tanaka 2002). How much a cell can dedifferentiate in a developmental pathway depends on several factors, such as type of cell, tissue of origin etc and is a subject of active research.

The 2012 Nobel Prize in Medicine was awarded to Shinya Yamanaka (alongside with Sir John B. Gurdon) "*for the discovery that mature cells can be reprogrammed to become pluripotent*", and, in particular, for the production of induced pluripotent stem

cells (iPS cells) from embryonic and adult fibroblasts (Takahashi and Yamanaka 2006; Takahashi, Tanabe et al. 2007). This achievement triggered a revolution in biology and in regenerative medicine. Over the last six years, researchers have been able to reprogram a wide variety of differentiated cells, through the ectopic expression of, so-called, reprogramming transcription factors, and achieve either the production of iPS cells or the transdetermination of "committed" cells, meaning the change of their differentiated state into a different one (Yu, Vodyanik et al. 2007; Eminli, Utikal et al. 2008; Park, Zhao et al. 2008; Stadtfeld, Brennand et al. 2008). Despite the vast amount of data that has been produced, it is still controversial whether reprogramming produces truly pluripotent stem cells with the capacity to generate a variety of differentiated cells (Hu, Weick et al. 2010; Kim, Doi et al. 2010; Bar-Nur, Russ et al. 2011).

Regeneration is a natural phenomenon during which the plasticity of differentiated cells is often challenged. Assaying how somatic stem cells are activated or differentiated cells reverse their fates in nature with the aim of proliferating and substituting the missing tissue can provide solid data about the actual developmental potential of different cell types.

In the following section, I will briefly present the history of regeneration research, describing the work that has been done in several animals across the phylogenetic tree. I will discuss basic mechanisms of regeneration and limb regeneration, in particular. I will, also, present the evidence that is available in the literature for the plasticity of the progenitor cells during regeneration. This will establish the foundation to describe basic aspects of appendage regeneration in *Parhyale hawaiensis*, discussing similarities and differences with other species. I want to introduce this way a new regeneration model system from the arthropods, a, relatively neglected taxon in regeneration studies. Finally, I will discuss the plasticity of progenitor cells during *Parhyale* appendage regeneration.

Introduction

Regenerative capacity can be broadly divided in three categories of descending complexity: (a) whole body regeneration, (b) structure regeneration, and (c) cellular regeneration.

Here are some examples:

(a) Planarians and *Hydra* are able to regenerate their whole body from almost any body part. If a flatworm, for example, is cut into five pieces, each of these pieces will give rise to a new worm.

(b) There are many different examples of structure regeneration. Many crustaceans can regenerate appendages lost to predators. The same is true for the limbs and the tails of salamanders. Zebrafish and newts can regenerate, amongst others, a big part of their heart. Humans and mice have also the capacity of regenerating their liver. Skeletal muscle, bone and epidermis are some of the mammalian tissues whose regeneration capacity has received extensive attention, especially because of medical implications.

(c) Cellular regeneration is the reconstitution of an injured cell. The most prominent example is axonal regeneration in the peripheral nervous system, which is observed in mammals. We can also place in this category regeneration of single-celled organisms, such as protozoans and the single-celled alga Acetabularia.

In the context of this thesis, I will refer to regeneration as the post-embryonic restoration of a body part that consists of a variety of different cell types, hence the first two situations.

2.1 History of regeneration research

Regeneration is truly a fascinating phenomenon. The first non-scientific reports of regeneration are found in Greek mythology. Lernaean Hydra was a mythological beast that was capable of regenerating two heads for every amputated one and Hercules had to burn the amputation stump in order to prevent this regeneration. Prometheus, an immortal Titan of Greek mythology was punished by Zeus to suffer an eternal torment; while bound to a rock, an eagle would eat his liver, which

regenerated within a day, and the torture would continue the next day. Although the myths are far from being true, the fact that Prometheus was reported to regenerate his liver and not for example his lung suggests that such regeneration events may have been observed and appreciated in the past.

The first thorough description of regeneration was in freshwater crayfish. In 1712, Rene Antoine Ferchault de Reaumur studied the "reproduction" of the limb in crayfish, where he noticed that "*nature gives back to the animal precisely and only that which it has lost, and she gives back to it all that it has lost*". He also described the successive stages of the regenerating limb bud, as well as the linkage between regeneration and molting (Réaumur 1712).

The discovery of Hydra regeneration was made by Abraham Trembley in 1744, presumably while trying to identify whether his "polyp" was a plant or an animal. Trembley sectioned the polyp in every possible way, transversely, longitudinally, or both, and into many pieces, eventually creating a multi-headed animal, which he named *Hydra,* after the Greek mythological beast. He also noticed the polarity of regeneration. *Hydra* regenerated a foot from the posterior and a head from the anterior part. Moreover, he mentioned that regeneration is faster in the anterior part of *Hydra* (Trembley 1744).

The first time regeneration was described in a vertebrate was in the second half of the 18th century, when Lazzaro Spallanzani observed in detail salamander tail regeneration. He studied the extent of regeneration compared to the extent of amputation, age- and species- dependent differences in regenerative capacity, the time course of regeneration. He closes his essay with the observation that regeneration follows unalterable laws independent of the number of amputations (Spallanzani, Maty et al. 1769).

Later, research in the field of regeneration identified the nerve dependency of limb regeneration in the newt (the neurotrophic phenomenon), described in 1823 by T. J. Todd; he amputated the sciatic nerve at the time of amputation, and observed the inhibition of regeneration (Todd 1823). Finally, in the late 19th century, Thomas Hunt Morgan tried to establish that regeneration resembles normal developmental processes (Morgan 1898). He experimented with hydromedusae, earthworms, planarians and

frogs, before changing directions and establishing *Drosophila* as a genetic model system.

2.2 Limb regeneration

During my PhD, I studied limb regeneration in *Parhyale hawaiensis*. Before describing the events that take place upon limb amputation in *Parhyale*, I will summarize what is known in other species that are able to regenerate their limbs. The main paradigms will come from amphibians, and especially newts, axolotls and frogs – tadpoles to be precise –, where the procedure has been meticulously examined.

The first response to amputation is wound healing. Initially, there is an inflammatory response accompanied by bleeding that represent the first line of defense against infection. Afterwards, the blood clots to prevent excessive bleeding (hemostasis), releasing matrix signals and growth factors. These factors enhance angiogenesis and migration of inflammatory cells (Martin 1997). The inflammatory cells are responsible for phagocytosis of any pathogens that invaded the wound and other cell debris. After a lag phase, epidermal cells start migrating to cover the wound surface, thus creating the wound epithelium. The initial events of limb regeneration, at least until the creation of the wound epithelium, resemble the wound healing procedure of non-regenerating skin. After the initial protection that is achieved by the aforementioned processes, the organism has to deal with the repair of the wound, which will lead either to the formation of a scar or to regeneration of the missing body part.

After covering the amputation surface, events that lead to regeneration of the tissue take place. Research on this phase of regeneration is still immature and not much information exists. The wound surface of the amputated limb seems to be important for regeneration to occur. The wound epithelium forms, upon activation by neural signals, the apical epithelial cap (AEC) (Satoh, Graham et al. 2008). Failure to form the AEC results in inhibition of limb regeneration (Thornton 1957; Satoh, Cummings et al. 2010). Also, the reorganization of the distal extracellular matrix through the action of specialized matrix metalloproteinases has been shown to be important for newt limb regeneration (Kato, Miyazaki et al. 2003). There is additional evidence for the importance of serine proteases, such as thrombin, in promoting cell-cycle re-entry of formerly quiescent cells in newt myotubes. It seems that in this case thrombin-

mediated cleavage generates an “activity” that is activating the dormant cells. Interestingly, mouse myotubes are resistant to this “activity” (Tanaka, Drechsel et al. 1999; Brockes and Kumar 2002). There have been reports of molecules that could mediate such signals, such as TGFb, BMP, FGF, Wnt etc (Antos and Tanaka 2010; Poss 2010).

Subsequently, cell proliferation can be detected in the regeneration blastema, in the tip of the limb. The blastema is an aggregation of morphologically similar proliferating mesenchymal cells, which gather underneath the epidermal cap and can participate in the regeneration of the missing structure.

Upon formation of the blastema, cells start acquiring a specific fate and differentiate ultimately contributing to the formation of the new structure. This starts with an outgrowth and expansion of the blastema and the subsequent patterning of this extension.

To achieve this, the differentiating cells need to know the segment to which they belong and their relative position along the proximodistal axis. How this happens is not clear. A plausible hypothesis is that the first blastema cells acquire distal positional values and the intermediate values are filled by intercalation of new blastema cells during regeneration (Echeverri and Tanaka 2005). A scenario that could explain how blastema cells acquire their positional identity is the existence of a distally located signaling center that creates a morphogen gradient, which provides the necessary positional information to the cells (Meinhardt 1983; Meinhardt 1983).

Once morphogenesis of the newly formed limb is complete, the regenerate is often smaller than the amputated limb. The limb then grows in order to acquire its final size. How this procedure is regulated, so that the new limb will have the appropriate size, has not received much attention and remains unclear.

The process described in this section reflects what is known so far from limb regeneration studies, mainly in axolotls. Many differences of opinion exist on the source and the cellular composition of the blastema, which is something that will be discussed later.

2.3 Regeneration in arthropods

Arthropods frequently lose appendages either to predators or due to complications while molting. Therefore, being capable to regenerate these appendages is probably crucial for survival. Members of all four major arthropod groups – Chelicerates, Myriapods, Crustacea and Hexapods - have been found to have good regenerative capacity (Maruzzo and Bortolin 2013). This does not mean that all arthropods have this ability; hence it is very interesting why some species lost this ability (assuming that the common ancestor of arthropods could regenerate). Moreover, the degree of regenerative capacity varies, with some species being able to regenerate their limbs completely, whereas others restore their appendages poorly or under specific conditions (e.g. developmental stage or amputation level) (Maruzzo and Bortolin 2013), adding an additional layer in the complexity of regenerative capacity.

Arthropods that can regenerate often bear a breakage point on the appendage, from which they can autotomize their limb. This is called autotomy plane and has probably evolved to help arthropods discard their appendage under the threat of a predator. However, not all arthropods that can regenerate are able to autotomize their appendages. Such ability has been also observed in starfish and lizards.

Another interesting phenomenon that has been reported both in arthropods and in amphibians is the nerve dependence of limb regeneration. It was shown both in Crustaceans and in amphibians that if the nerve that innervates the limb is cut proximally to the amputation point soon after amputation, then regeneration was affected (inhibited in the case of salamanders and retarded and incomplete in the case of crustaceans) (Needham 1946; Singer 1952). Nerve dependence might have evolved in order to ensure the concurrent regeneration of the nerve alongside with the rest of the structure (Kumar and Brockes 2012).

Another important aspect of regeneration in arthropods is the relationship between molting and regeneration. Many arthropods molt in their entire lifetime and molting is coupled with growth. Moreover, the regenerate is revealed after the following molt. Amputation and subsequent regeneration has been shown to affect the timing of molting in different species – delaying or accelerating it (Maruzzo and Bortolin 2013). Moreover, a critical point of the intermoult period has been observed after which an appendage cannot regenerate before the subsequent molt. This critical point

in crickets has been associated with an ecdysteroid peak (Maruzzo and Bortolin 2013).

Four "model" species have been used to study arthropod appendage regeneration in greater detail: *Gryllus* (cricket), *Blatella* (cockroach), *Tribolium* (beetle) and *Drosophila* (fruitfly).

Crickets, like cockroaches, are capable of regenerating a fully functional nymphal leg. Studies in cockroaches had indicated the importance of proximodistal and circumferential positional identity leading to the proposal of the polar coordinate model (French, Bryant et al. 1976). However, the molecular basis was still unknown. With the use of nymphal RNAi in crickets, it was shown that Wnt, BMP and EGF pathways participate in the acquisition of proximodistal positional identity by the cells (Nakamura, Mito et al. 2008). Likewise, the importance of Wnt signaling was also highlighted during larval leg regeneration in *Tribolium* (Shah, Namigai et al. 2011). Further studies in *Tribolium* have also indicated the requirement of matrix metalloproteinases in wound healing during larval leg regeneration (Mitten, Jing et al. 2012).

Studies in *Drosophila* focus mostly on imaginal disks, where regeneration is occurring. Since *Drosophila* is the most studied model arthropod, it is worth having a look on the way imaginal disk regeneration occurs. After wound healing, a blastema is formed, which grows and is then being patterned to restore the missing part (Bryant and Fraser 1988). When a disk is injured in a region called "weak point", during regeneration some blastema cells can lose their identity and regenerate structures normally formed by different disks, e.g. leg disk cells may form wing structures and antennal cells may form leg structures (Sustar and Schubiger 2005). This process is called transdetermination (Garcia-Bellido 1966).

2.4 Basic mechanisms of regeneration

Regeneration can proceed via three different mechanisms in terms of origin of new tissues. (a) The activation and proliferation of totipotent adult stem cells; this is the main way of regeneration in planarians. (b) The proliferation of lineage-restricted progenitor cells; mainly described in the vertebrate regeneration models. (c)

Transdifferentiation, i.e. the transformation of differentiated cells into different cell types.

2.4.1 Pluripotent adult stem cells in planarians

The mechanism of restoring the missing tissue via totipotent adult stem cells has been described in flatworms. As mentioned earlier, planarians are capable of regenerating the whole body from a small fragment. This proceeds by the formation of a blastema in the wound site, where the missing tissue is generated. The blastema is formed by somatic stem cells, which are abundant in the parenchyma of the planarian body and are called neoblasts. The importance of neoblasts in regeneration is highlighted by the fact that their absence in the anteriormost part coincides with the inability of planarians to regenerate a whole animal only from the anterior part of the head (Newmark and Sanchez Alvarado 2000; Reddien and Sanchez Alvarado 2004). Moreover, irradiation of the planarians, which results to the death of the neoblasts, abolishes their capacity to regenerate (Lange 1968; Reddien, Oviedo et al. 2005).

However, until recently, it was not known whether the neoblasts represent a homogeneous population of cells or they can be separated to subpopulations with different developmental potential. By combination of irradiation and transplantation, it was rigorously proven that all regenerated cell types can come from a single type of totipotent cell, termed clonogenic neoblast, which can ultimately give rise to all the different tissues of the flatworm (Wagner, Wang et al. 2011). It remains to be identified how many of the neoblasts are actually clonogenic. It is also interesting to note that the neoblasts are not dormant, even under non-regenerative conditions. Instead, they are responsible for the homeostasis of aging tissues.

It has yet to be determined how clonogenic neoblasts differentiate and give rise to their descendants. We still do not know whether this happens via initial differentiation to intermediate proliferative committed neoblasts or if clonogenic neoblasts generate directly differentiated cells.

2.4.2 Totipotent, lineage-restricted progenitors and transdifferentiation in Cnidarians

Hydra is a simple animal consisting of a head and a foot which are connected with a body column. It consists of two epithelial layers, one of endodermal and one of ectodermal origin – *Hydra* is diploblastic. The three distinct stem cell populations are

the following: (a) interstitial stem cells that are situated in the interstices between the ectodermal and endodermal epithelial cell layers. They are multipotent stem cells that regenerate nerves and nematocytes (David and Murphy 1977). (b) The ectodermal and (c) endodermal cells are distinct stem cell populations that proliferate to repopulate the respective epithelial layers (Bosch 2007).

How committed and how homogeneous each of these stem cell populations is, remains to be studied. Also, transdifferentiation has been observed without, however, crossing the boundaries of each germ layer. It has been reported that zymogen cells can transdifferentiate in order to generate granular mucous cells during head regeneration (Siebert, Anton-Erxleben et al. 2008). Both cell types are endodermal.

Hydractinia polyps resemble *Hydra* polyps in morphology. However, they differ in terms of progenitor cell plasticity. Their interstitial stem cells (termed i-cells) are totipotent. Transplantation of i-cells from a donor animal into i-cell-eliminated recipient animals (of different sex, growth pattern and morphology) led to the gradual conversion of the genotype and the phenotype of the recipients into the genotype and phenotype of the donors (Muller, Teo et al. 2004).

2.4.3 Lineage-restricted progenitors and transdifferentiation in vertebrates

Lineage-restricted progenitor cells have been observed in regeneration of vertebrates' appendages and organs. Most information comes from Amphibia. Lately, zebrafish has emerged as a potent regeneration model; they can regenerate their fin, heart and other tissues. Vertebrates regenerate via the formation of a blastema. Blastema was initially considered as collection of indistinguishable mesenchymal cells that contributed to regeneration. The composition of the blastema was until recently not known. The regenerative potential of these mesenchymal cells, i.e. whether each can give rise to diverse tissues, and their origin, meaning whether they come from differentiated cells that dedifferentiate or from stem cells, has been an area of active research the last ten years.

Xenopus tadpole can regenerate its tail. In the tail, one can distinguish the muscles, the notochord and the spinal cord, each of which derives from a different lineage. By transplantation of labeled cells in a non-labeled animal, it was shown that progenitor

cells contributed to the regeneration of structures within their lineage and did not participate at all to the regeneration of others (Gargioli and Slack 2004).

Contrary to the situation in the tadpole, during regeneration of the axolotl (*Ambystoma mexicanum*) tail, the distinction between ectodermal and mesodermal progenitors is not so precise. Using single-cell electroporation of neural precursors of the spinal cord, it was shown that ectodermal cells could occasionally change their fate and contribute to the regeneration of cartilage and muscle, which is of mesodermal origin (Echeverri and Tanaka 2002). Nevertheless, the occurrence and/or the frequency of such events has been challenged (McHedlishvili, Epperlein et al. 2007).

Contrary to what is observed during tail regeneration, axolotl limb regeneration occurs with robust lineage restriction. Using skin, cartilage and embryonic presomitic mesoderm grafts from ubiquitously labeled animals to unlabeled ones, mosaic animals were generated expressing GFP in specific tissues (dermis and epidermis, cartilage, muscle and satellite cells, respectively). It was shown that after regeneration, muscles had only given rise to muscle, and not cartilage or epidermis, cartilage had regenerated cartilage, but not muscle, and dermis was the most flexible lineage having formed cartilage and connective tissue, but not muscle (Kragl, Knapp et al. 2009). These results show that in an axolotl limb, regeneration occurs from committed progenitor cells, indicating that axolotls might utilize different mechanisms to regenerate their tails and their limbs.

Newts (*Notophthalmus viridescens*) are capable of regenerating many different tissues, such as limbs, the tail, the brain, the lens, the retina, and the heart (Sanchez Alvarado and Tsonis 2006). The regeneration of the lens presents an interesting phenomenon. Their regenerating lens is formed by the dedifferentiation of dorsal iris pigment epithelial cells and their transdifferentiation into lens cells. What is surprising is that the ventral iris pigment epithelial cells are not capable of transdifferentiating into lens cells (Del Rio-Tsonis and Tsonis 2003).

Zebrafish (*Danio rerio*) is a powerful model for regeneration studies. It can regenerate its fins, tail, heart, liver, spinal cord, hair cells of inner ear and lateral line, while, also, many genetic tools are available. By creation of transposon-based clones, a highly restricted pool of progenitors was identified. Specifically, nine distinct lineages were

recovered, each of which contributed to regeneration of structures within the same lineage during fin regeneration, namely epidermis, melanocyte/xanthophore, iridophore, intraray glia, lateral line, osteoblast, dermal fibroblast, vascular endothelium, and resident blood lineages (Tu and Johnson 2011). At the same time, it was shown, using Cre/lox technology, that mature osteoblasts regenerated osteoblasts by dedifferentiating to an immature state, proliferating, migrating to the blastema and giving rise to new osteoblasts (Knopf, Hammond et al. 2011).

Mammals have limited regenerative capacity. Digit-tip regeneration, liver and pancreas regeneration have been studied regarding their regeneration progenitors. During digit-tip regeneration in mice, germ layer restricted progenitors are responsible for the regeneration of the ectodermal and mesodermal tissues. Moreover, there is further restriction within germ layers in different pools of progenitors for dorsal ectoderm, ventral ectoderm, tendon, bone and blood vessel lineages (Rinkevich, Lindau et al. 2011). After hepatectomy, the regeneration of the lost liver part occurs by proliferation of the mature cells, without apparent dedifferentiation or transdifferentiation (Sanchez Alvarado and Tsonis 2006). Under persistent liver injury, bipotent hepatic progenitor cells are activated to generate hepatocytes and biliary epithelial cells (Zhao, Ren et al. 2009). During pancreas regeneration, transdifferentiation of a-cells to beta-cells has been shown to occur after total beta-cell loss, phenocopying diabetes (Thorel, Nepote et al. 2010). Moreover, other pancreatic epithelial cells (such as duct or acinar cells) have been proposed to have the capacity to transdifferentiate into beta-cells (Lysy, Weir et al. 2013).

Results - Discussion

2.5 General observations on *Parhyale* appendage regeneration

Since it is the first time regeneration is being studied in *Parhyale*, I initially describe some basic aspects of appendage regeneration in *Parhyale*, comparing my observations to what is known in other arthropods.

Parhyale can regenerate its antennae, maxillipeds, thoracic legs (T2-T8) pleopods and uropods. All the experiments in my thesis have been performed in antennae and thoracic appendages for practical reasons; they are larger, therefore easier to observe. Appendage regeneration seems to be very important for survival, since appendages can be lost during molting, which happens regularly through *Parhyale*'s entire lifetime. On the other hand, *Parhyale* cannot regenerate its primary body axis. So, even if a small part in the pleon is amputated, it cannot be replaced. *Parhyale*'s capacity of regeneration is placed in the second of the categories that are mentioned in the Introduction. We haven't assessed whether *Parhyale* can regenerate any of its internal organs.

There is no autotomy during regeneration in *Parhyale*. Wherever the amputation occurs, regeneration starts slightly more proximally from the amputation point. However, there seems to be a preferred breakage point, because most of the times when natural amputation was observed (by natural, I refer to non-experimental amputation), the amputation point was in the most distal part of the basis, close to the ischium. Similarly, other amphipods, such as *Orchestia*, have a preferred breakage point at the same site on the appendage between basis and ischium (Charniaux-Cotton 1957).

Parhyale regenerates its appendages very effectively generating a perfect replica of the missing part (Figure 15). The thoracic and abdominal appendages, when regenerated, have the same number of segments and are indistinguishable from appendages before amputation. Occasionally, when assaying antennal regeneration, I have observed that the newly formed antenna might bear fewer segments. However, this is not observed often and is generally corrected after subsequent molts. Moreover,

the antennae in *Parhyale* differ from the thoracic appendages in that they don't have a defined number of segments.

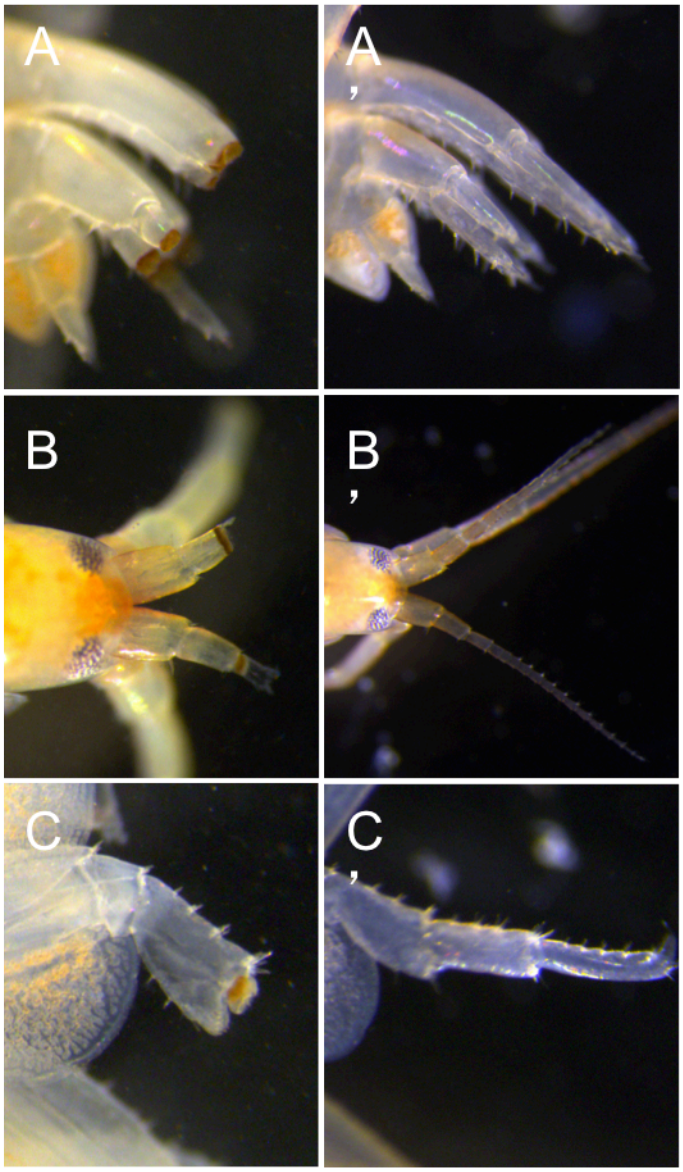


Figure 15: *Parhyale* can regenerate all of their appendages
Parhyale can regenerate all of their appendages within one moulting cycle. (A-C) Amputated abdominal (uropods), cephalic (antennae 1) and thoracic appendages (T6). (A'-C') After the next moult, these appendages have fully regenerated.

In order to probe whether regeneration is complete at the level of individual tissues and cell types, I used three transgenic lines that were available in the lab. These lines express DsRed in epidermal cells (Distal-DsRed line), mesodermal cells (MS-DsRed line) and nerve cells (DC5-DsRed) in *Parhyale* appendages.

Using animals from the DC5-DsRed line, I was able to show that the nerves that express DsRed under the DC5 promoter regenerate properly (Figure 16A-A'). Also, using mosaic animals expressing EGFP in the ectoderm (described in section 2.9), as well as the Distal transgenic line that expresses DsRed in the epidermis with increasing intensity when moving from proximal to distal I was able to verify that ectodermal cells regenerate precisely (Figure 16B-B'). Finally, I amputated appendages in an animal that expresses DsRed under the *Parhyale* muscle-specific promoter (Pavlopoulos and Averof 2005). The muscle composition of the appendage before and after regeneration is practically indistinguishable, contrary to what has been observed in cockroaches (Kaars, Greenblatt et al. 1984). Moreover, as I will illustrate in Chapter 3, I identified that this promoter drives DsRed expression in all the mesodermal cells of the limbs and not only the muscles. Therefore, I can conclude that all the mesodermal tissues I could distinguish regenerate accurately (Figure 16C-C').

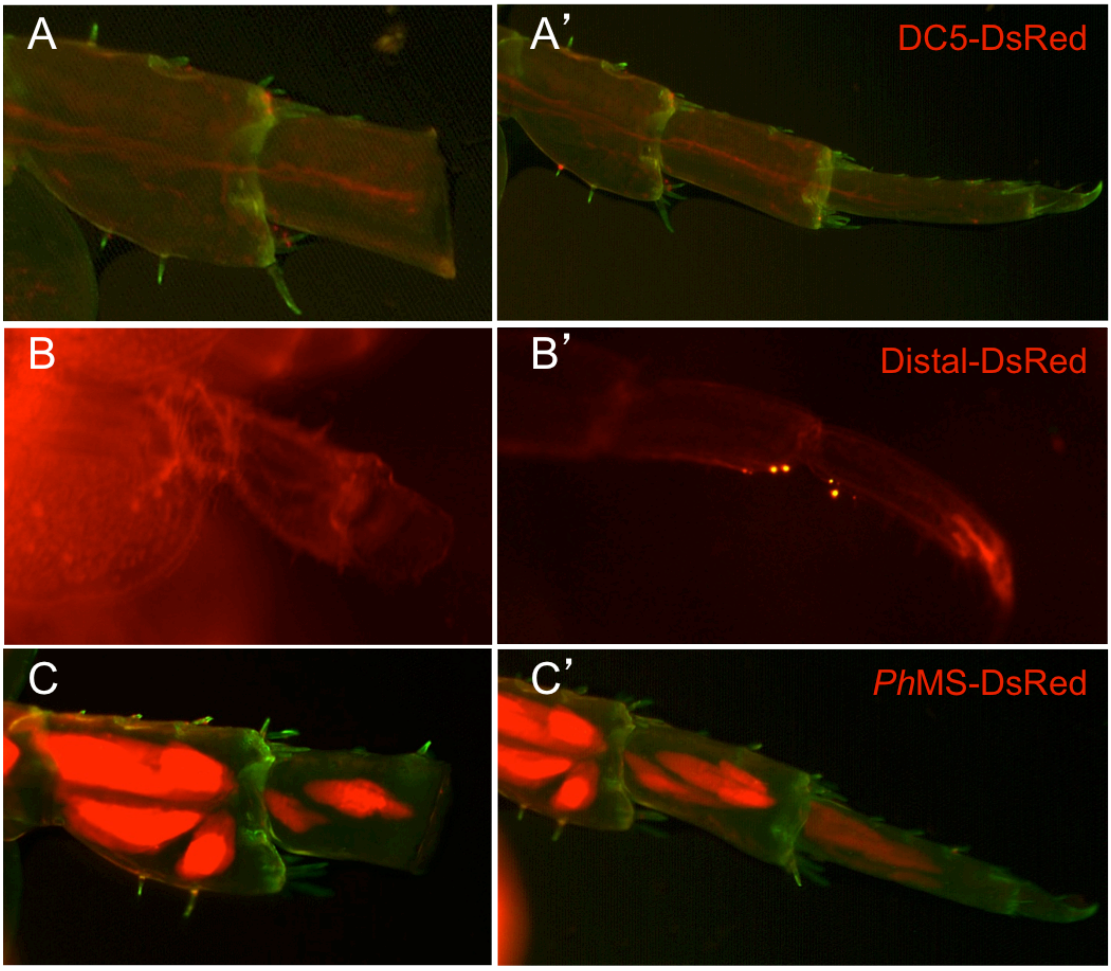


Figure 16: *Parhyale* can regenerate multiple cell types of the limb
By using different transgenic markers, I was able to show that *Parhyale* can regenerate muscle, nerves and epidermis. (A and A') Images after amputation and after regeneration of a thoracic appendage belonging to an animal of the DC5-DsRed line, expressing DsRed in a nerve that traverses the appendage. Autofluorescence of the cuticle can be seen in green in all images. (B and B') Images after amputation and after regeneration of a thoracic appendage showing DsRed expression in the epidermis. The animal is from the *Distal*-DsRed line, where DsRed is expressed in the epidermis with strongest expression in the distal part. (C and C') A thoracic appendage from an animal from the *Ph*MS-DsRed line was amputated and left to regenerate.

I observed that the muscles of the regenerating leg usually regenerate close to the next moult, after the regeneration of the epidermis (Table 2). As was mentioned in the first Chapter, during embryogenesis mesoderm relies on ectoderm for segmentation (Hannibal, Price et al. 2012), which might be the case during regeneration and the reason why mesoderm regenerates after ectoderm.

a/a	amputation	moult	first muscle regeneration	complete muscle regeneration
1	0	7	9	11
2	0	9	10	14
3	0	9	13	15
4	0	10	13	15
5	0	11	13	16
6	0	12	12	17
7	0	12	14	17
8	0	17	21	-
9	0	23	26	32
10	0	24	27	-
11	0	25	23	30
12	0	26	31	37
13	0	29	33	41

Table 2: Muscles regenerate slightly after the moult that reveals the newly-formed appendage
Timing of muscle regeneration with respect to the moult that reveals the newly-formed appendage (in days post amputation).Each row corresponds to an individual where two thoracic limbs were amputated. "First muscle regeneration" refers to the first appearance of *Ph*MS-DsRed; "complete muscle regeneration" refers to the complete recovery of *Ph*MS-DsRed expression as judged by comparing the regenerated limb to an unamputated one.

Parhyale can also regenerate the same appendage over and over again. Amputating the same appendage even six times successively does not have any effect in its regenerative capacity. Also, *Parhyale* can regenerate all of their appendages independent of their age. *Parhyale* can live even up to two years. I assayed animals that were up to 18 months old for their regenerative capacity and they were able to regenerate thoracic appendages and antennae.

Very rarely, malformations (abnormal regenerates) have been observed (Figure 17). This happens only when the amputation is very proximal and it is always corrected after the next molt.

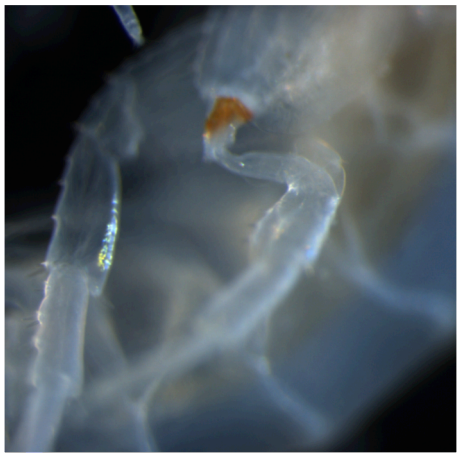


Figure 17: Incomplete regeneration
In few cases, incomplete regeneration may be observed. This occurs mainly when the appendage is amputated late too soon before the moult and the appendage does not have sufficient time to regenerate properly before moulting. In all the cases, the appendage regenerates properly after the subsequent moult.

Appendage regeneration occurs within the cuticle of the amputated limb and the new limb is revealed when the old exoskeleton is discarded. The regenerate is usually complete by the time of the first molt. On occasions when amputation occurs close to the next molt, the new appendage is completed after the second molt. The relationship between molting and regeneration will be discussed in the next section. Since regeneration is taking place within the cuticle, it is not trivial to observe the whole procedure under white light.

I have used two ways to record regenerative events within the exoskeleton. The first requires fixation of the animal and is, therefore, incompatible with live inspection of regeneration. Using nuclear staining with TOPRO, I have obtained still images of regeneration at different time points after amputation. The advantage of this approach is that it marks every nucleus, giving the opportunity to have a complete overview of regeneration (Figure 18A). The second approach was to use transgenic animals that express fluorescent proteins in specific cells. I used transgenic animals of the Distal line (Kontarakis, Pavlopoulos et al. 2011), which express DsRed mainly in the distal part of the appendages. This way, I was able to record regeneration from the formation of the distalmost part of the appendage onwards (Figure 18B).

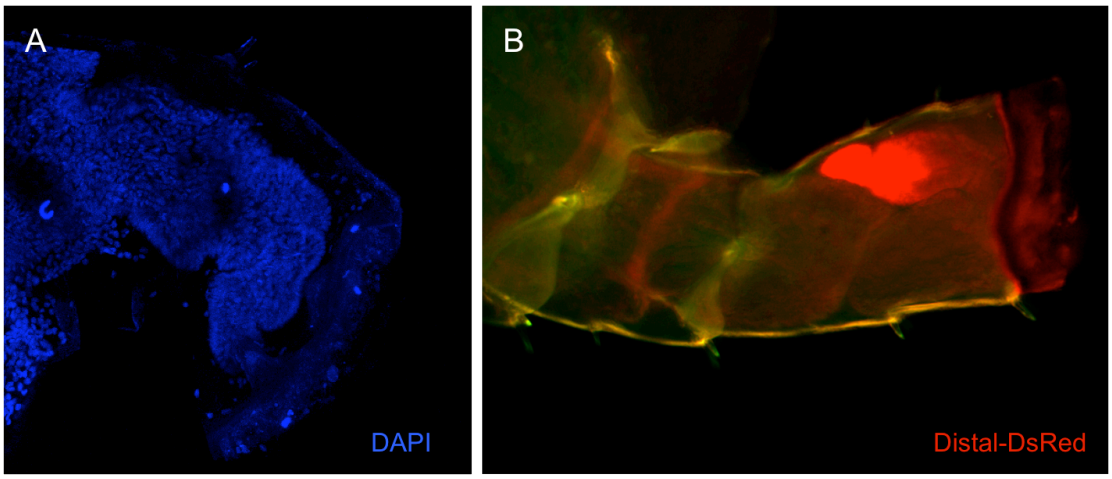


Figure 18: Ways to visualize regenerating appendages within the old exoskeleton
(A) Nuclear staining with DAPI of the regenerating appendage can reveal the outline of the appendage that has been formed. The disadvantage of this method is that it is not compatible with live-imaging since it requires fixation of the tissue. (B) Using transgenic reporters or gene traps we able to visualize regeneration and to study the behavior of the marked cells in real time. In this case, a regenerated appendage of an animal from the Distal-DsRed line can be seen. The regenerated appendage is bent to occupy the available space within the old exoskeleton.

Using this line, I was able to recognize two distinct phases in *Parhyale* appendage regeneration. In the first phase, the animal regenerates a small replica of the missing appendage and, in the second, the newly formed appendage grows in size.

2.6 Correlation between regeneration and molt

I wanted to understand how the speed of regeneration varies among individuals. I assayed the timing of regeneration by the first appearance of Distal-DsRed expression. By examining many different animals, it was easy to conclude that the duration of regeneration varies a lot among different animals, ranging from three to ten days post-amputation. Following, I wanted to know whether regeneration was somehow connected with molting, being influenced by the molting cycle or being regulated similarly with molting, for example by ecdysteroids, as discussed in the Introduction. In order to resolve this putative correlation and, also, because it would be useful for subsequent experiments for me to be able to estimate the timing of regeneration, I performed the following experiments using animals from the Distal line.

I, initially, wanted to see whether there was any correlation between the duration of regeneration and the remaining time until the subsequent molt. Although there was a positive correlation, this was not robust (Figure 19).

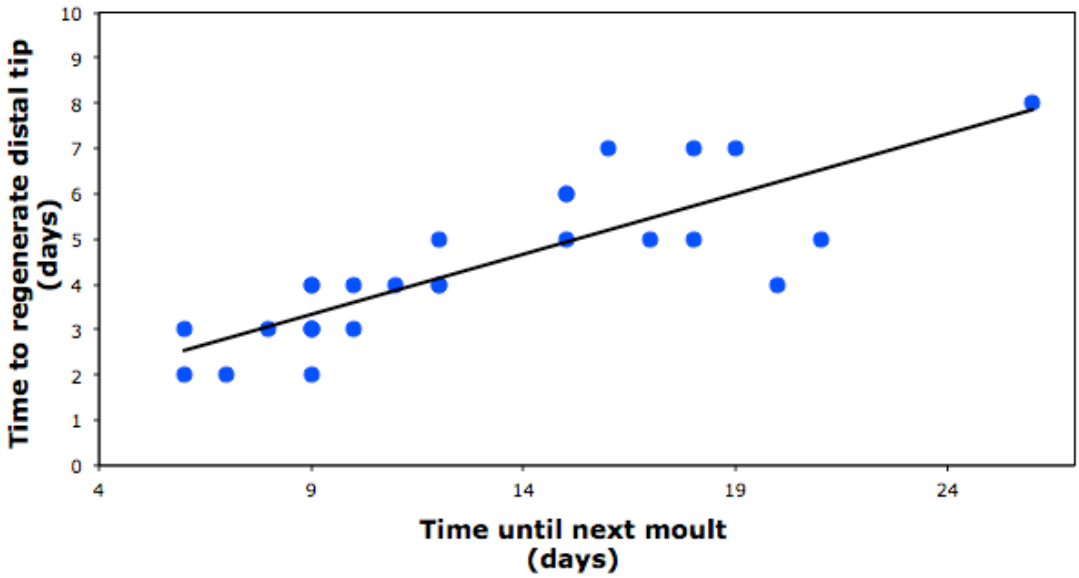


Figure 19: Regeneration time and time remaining until the subsequent moult are loosely correlated
The time an appendage needs to regenerate within the old exoskeleton was recorded by the expression of DsRed in the distal part of the appendage, in animals from the Distal-DsRed line. Two thoracic appendages from individuals of different ages were amputated and left to regenerate. The first appearance of DsRed in the tip of the appendage, as well as the time of the subsequent moult, was recorded. Each data point represents one scored animal. The time needed to regenerate the distal tip is loosely correlated to the timing of the subsequent moult.

Subsequently, I examined whether there is a correlation between the duration of regeneration and the time elapsed from the previous molt. The result in this case showed no correlation between the previous molt and the regeneration timing (Figure 20).

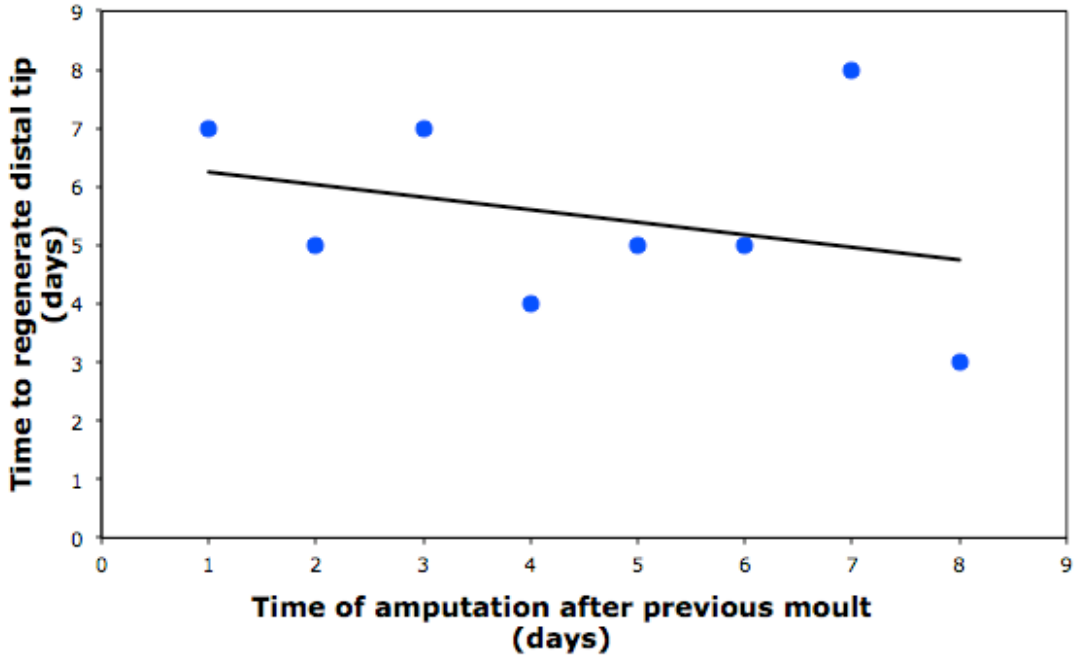


Figure 20: Regeneration time and time elapsed from the subsequent moult are not correlated
Animals of different ages from the Distal-DsRed line were collected and kept separately. Their moult was recorded and thoracic appendages from each one were amputated in different time points after the moult. The timing an appendage needs to regenerate within the old exoskeleton was recorded by the expression of DsRed in the distal part of the appendage. Each data point represents one scored animal. The time needed to regenerate the distal tip is not correlated to the time elapsed from the previous moult.

Finally, I assayed the relationship between the duration of the intermolt period (from one molt to the next) and the duration of regeneration and found positive correlation. Using animals of different age and having a range of intermolt period lengths, I observed that the duration of regeneration was proportional to the duration of the intermolt period. Specifically, the period between amputation time and the onset of Distal-DsRed expression is approximately a quarter of the intermolt period (Figure 21). This means that if, for example, the intermolt period lasts twenty days, it takes the animal five days to regenerate an appendage up to the stage where Distal-DsRed expression is timed on.

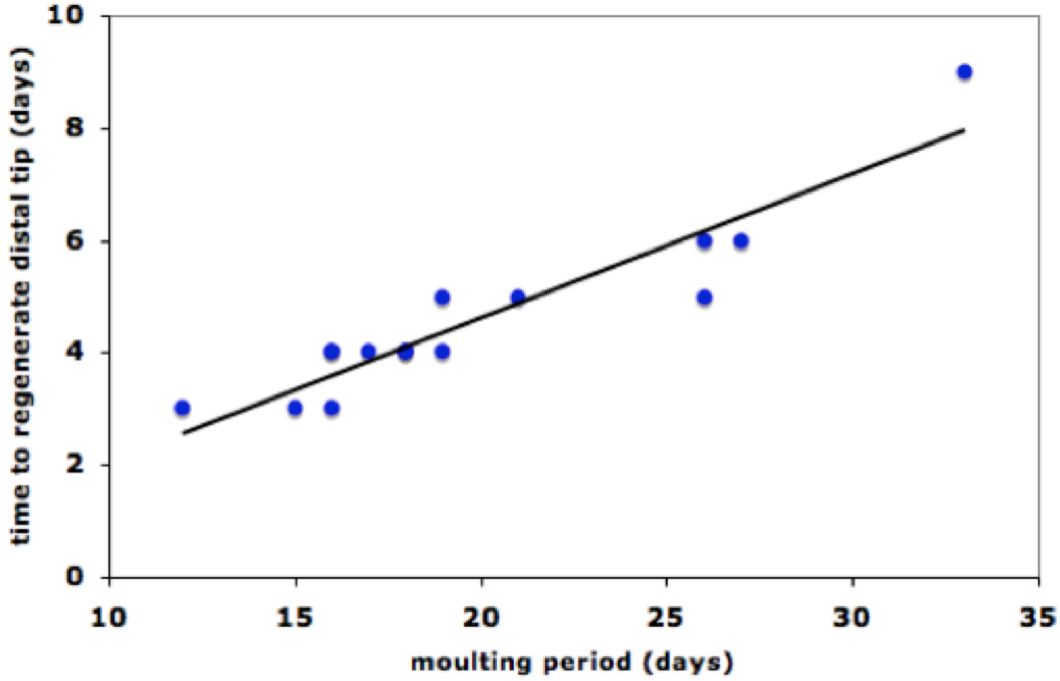


Figure 21: Regeneration time and the length of the intermoult period are correlated
The timing an appendage needs to regenerate within the old exoskeleton was recorded by the expression of DsRed in the distal part of the appendage, in animals from the Distal-DsRed line. Two thoracic appendages from individuals of different ages were amputated and left to regenerate. The first appearance of DsRed in the tip of the appendage was recorded and plotted against the length of the intermoult period, i.e. the time from the moult preceding to the moult following the amputation. Each data point represents one scored animal. The time needed to regenerate the distal tip is strongly correlated to the moulting frequency, which is in turn influenced by age, health and nutrition.

This result suggests that molting and regeneration are dependent upon similar physiological conditions. We know that molting is coupled with growth and, therefore, the intermolt period is shorter in young, healthy and well-fed animals. This could also mean that the younger, healthier and better fed the individual is, the faster it regenerates. Moreover, we are now able to estimate the approximate duration of regeneration by measuring the intermoult period.

2.7 *Parhyale* appendage regeneration

Based on the previous results I was able to divide regeneration procedure from amputation to exposure of the regenerate in five stages, relatively distinct from each other, which I will try to describe in this section. The five phases are: (a) wound closure, (b) blastema formation, (c) morphogenesis, (d) growth, (e) lag phase and molt. Figure 22 shows the timeline of *Parhyale* appendage regeneration in a young (~6 month old) healthy individual.

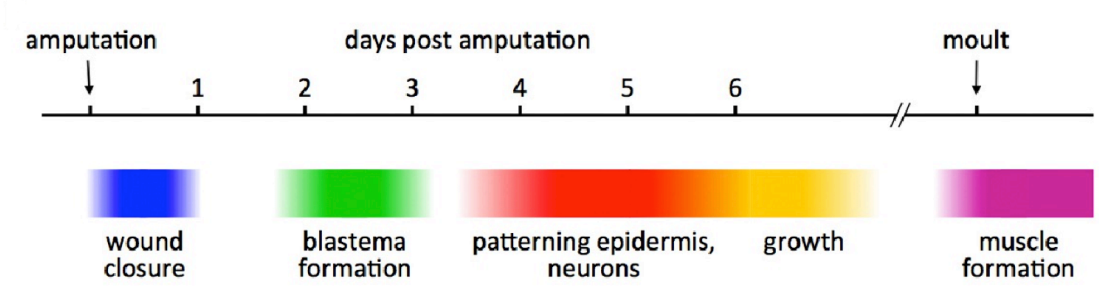


Figure 22: Timeline of *Parhyale* appendage regeneration
The course of events that happen after appendage amputation in a young (~6 month old) healthy individual is indicated. Initially, during the first day post amputation, the wound closes forming a melanized scab. Subsequently, the blastema is formed. This happens within the first two-three days post-amputation. Then, the neurons and the epidermis of the appendages are patterned (the patterning of the epidermis, visualized by Distal-DsRed, can vary between three and nine days – see Figure 21) before they grow to acquire their final size. Finally, differentiated muscles appear shortly after the moult (muscle regeneration, visualized by *Ph*MS-DsRed, can be completed within 4-12 days after moult – see also Table 2).

(a) Upon amputation, the first thing that happens is the formation of a melanized scab on the wound surface. The scab is connected to the old exoskeleton. So, when the animal molts, independently of whether the appendage had time to regenerate or not, the scab is removed alongside with the old exoskeleton. This stage lasts approximately 24 hours; images from different time-points of this stage can be seen in Figure 23.

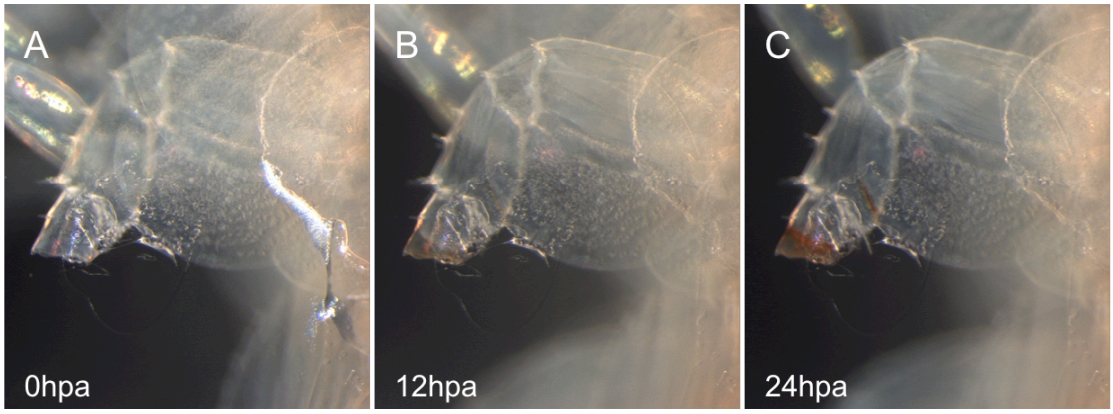


Figure 23: Wound closure and melanized scab formation
Stills from a video that was taken during the first day after amputation show the formation of the wound epithelium in the distal-most part of the amputated appendage. hpa: hours post-amputation

(b) The second phase is characterized by the formation of a blastema, an accumulation of proliferating cells in the distalmost part of the severed appendage. This phase is not distinct from the wound closure and it can also be considered as the

beginning of the morphogenesis . The proliferation of the cells starts approximately twelve to eighteen hours post-amputation and is completed within two or three days. The proliferation and the accumulation of the proliferative cells in the distalmost part of the appendage can be detected with EdU staining (Figure 24).

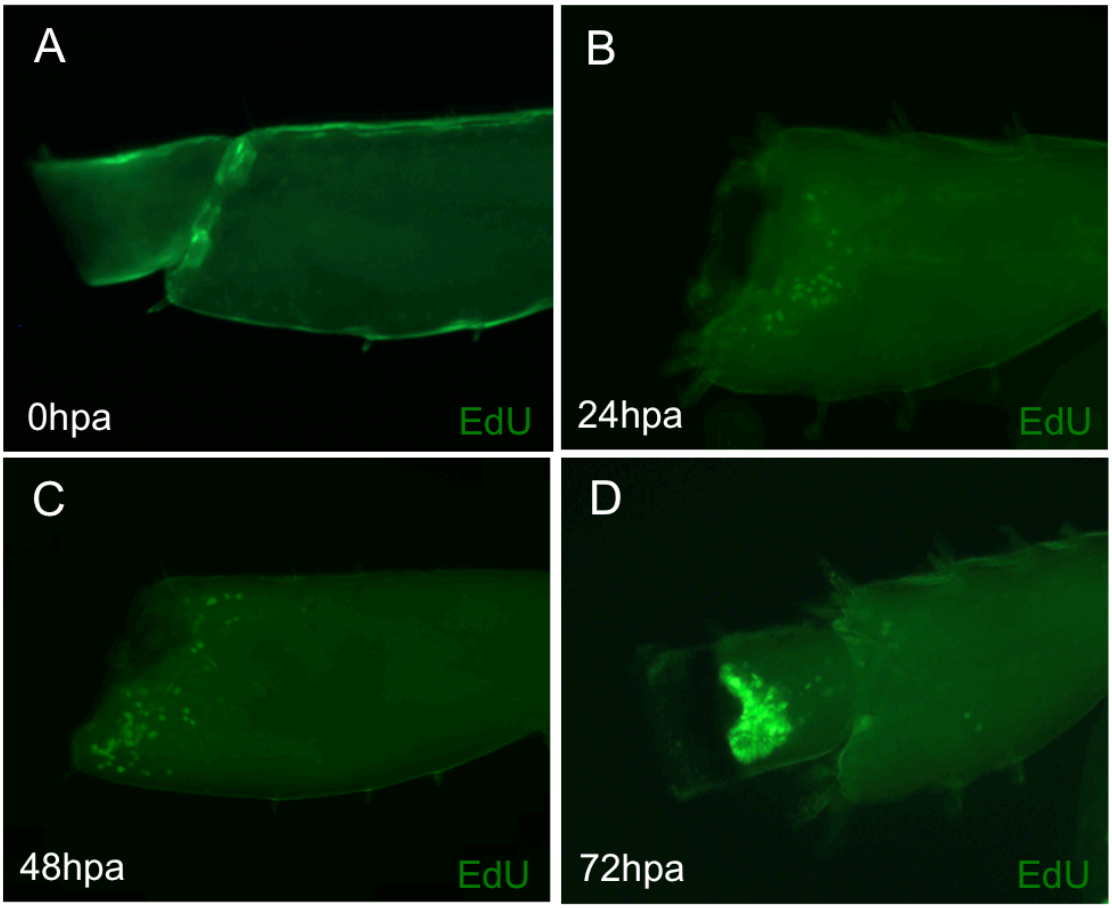


Figure 24: Blastema formation during leg regeneration
Within 2-3 days after amputation proliferating cells accumulate underneath the wound epithelium forming the blastema. Cells that have undergone proliferation are marked here by EdU incorporation (green). 12 hours before staining, 0.2mM EdU in seawater was administered to the animals for 6 hours, the animals were then kept in seawater for another 6 hours. Their amputated appendages were then fixed and stained. hpa: hours post-amputation

(c) After the formation of the blastema, we can observe, in the distal end of the amputated appendage, the regenerate acquiring its characteristic morphology of the appendage. This phase varies in length. In this period, the tissue establishes a distal region, as can be observed by the expression of Distal-DsRed in Figure 25, and then the new appendage is formed. The regenerate at the end of this phase seems to be complete but it is still small. Figure 25A-C illustrates representative stills from a video, which records the morphogenesis phase of regeneration of an animal from the

Distal line. Initially, DsRed can be observed weakly in the whole appendage. Later, DsRed expression is upregulated in the distal part of the amputated limb, and, shortly after, the tissue starts acquiring the configuration of an appendage. In Figure 25D, you can also see the EdU staining by the end of this phase.

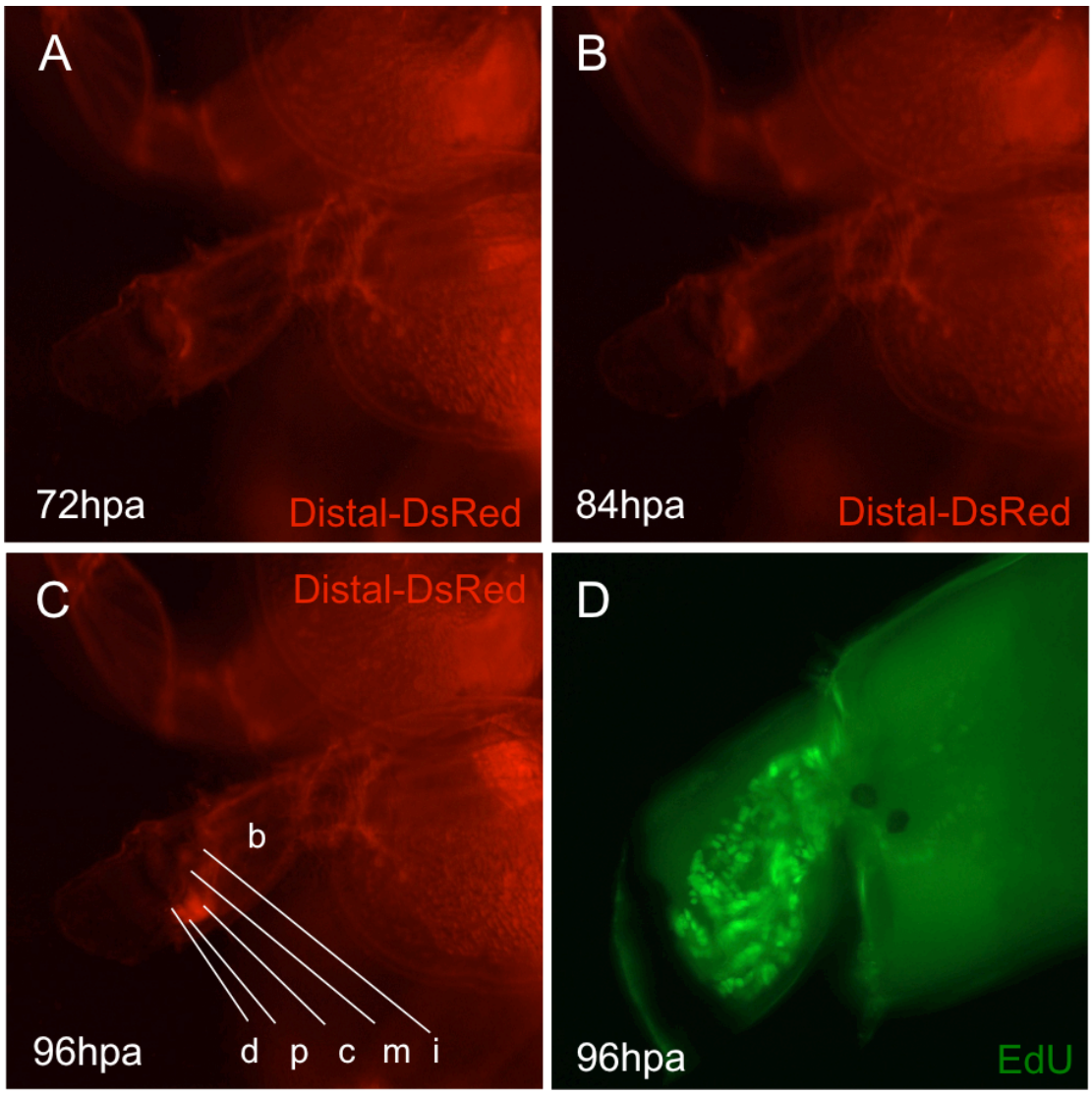


Figure 25: Patterning of the regenerating appendage
(A-C) Stills from a video that shows the regeneration of the sixth thoracic appendage of an animal carrying the *Distal*-DsRed gene trap. During the fourth day after amputation, the appendage is patterned acquiring its final shape and all its podomeres (indicated in (C); b, basis; i, ischium; m, merus; c, carpus; p, propodus; d, dactylus). By the end of this stage, the leg is patterned but significantly smaller than the unamputated appendage. DsRed is expressed in epidermal cells, most strongly in the distal part of the appendage. (D) Staining of proliferating cells with EdU by the end of this stage. As earlier, 12 hours before staining, 0.2mM EdU in seawater was administered to the animals for 6 hours, the animals were then kept in seawater for another 6 hours; then, their amputated appendages were fixed and stained. The leg can be seen bent within the old exoskeleton. hpa: hours post-amputation

(d) After the new appendage is formed, it grows to acquire the appropriate size. This phase is characterized by extensive proliferation leading to the extension of the limb, which bends within the old exoskeleton due to restricted space. In Figure 26A-C, one can see stills from the same movie at later stages. The growth of the limb is evident. Moreover, EdU staining shows the extent of proliferation in order for the limb to acquire it final size (Figure 26D). It would be interesting to know how proliferation and, hence, final size is regulated. It is clear that an appendage of the wrong size, smaller or bigger, would be a great disadvantage for the individual.

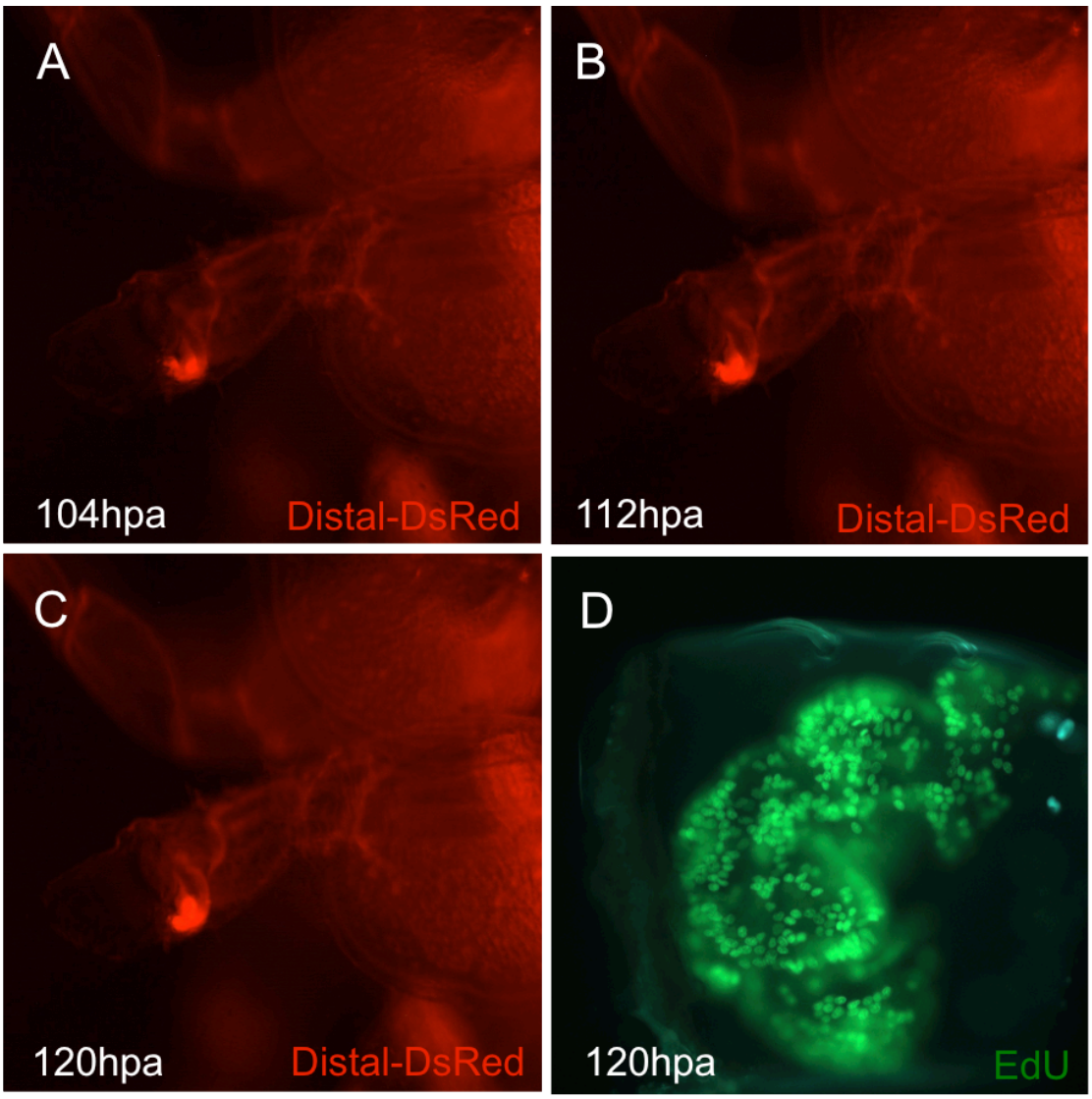


Figure 26: Growth of the regenerating appendage
(A-C) Stills from the same video as in Figure 25. In the last stage, the appendage grows prior to moulting. (D) Staining of proliferating cells with EdU 5 days after amputation. EdU staining was performed as described previously. hpa: hours post-amputation

(e) During this phase, no changes can be observed. The regenerate is already structured and has acquired the proper size and just "waits" to be revealed. It is often the longest among the five phases.

2.8 Effects of Ubx overexpression on regeneration

Hox genes play a pivotal role in positional identity during embryogenesis. Misexpression of Hox genes in the embryo leads to severe transformations of one body part into another, termed homeotic. Moreover, lately, it has been demonstrated that Hox genes are also regulated tightly in adult tissues through epigenetic mechanisms (Schuettengruber, Chourrout et al. 2007).

It is very interesting to assess the role of Hox genes during regeneration. Are Hox genes expressed in the same pattern in adults as they are in embryos? Are they the genes that provide segmental identity during regeneration? If a Hox gene is misexpressed during regeneration will it have an effect on the regenerate? Is this effect permanent, does it change the epigenetic regulation of the gene? Reports from other species have been scattered and inconclusive. In *Xenopus*, the same genes are expressed in embryonic tail buds and in the regenerating tail blastema (Christen, Beck et al. 2003). In planarians, newts and *Platynereis*, there are Hox genes expressed during regeneration but their expression is regeneration-specific (Simon and Tabin 1993; Bayascas, Castillo et al. 1998; Pfeifer, Dorresteijn et al. 2012).

I decided, in collaboration with Zacharias Kontarakis, to assess the effect of Ubx in regenerating appendages. We already knew that Ubx expression is responsible for the embryonic development of locomotory appendages (T4-T8) and misexpression of Ubx in more anterior segments led to the transformation of the antennae and gnathopods to locomotory limbs (Pavlopoulos, Kontarakis et al. 2009).

Initially, we wanted to examine whether Ubx is expressed in the adult appendages and whether the adult expression pattern resembles the embryonic one. Therefore, we isolated mRNA from thoracic appendages (T1-T4) and cephalic appendages (antennae 1 and antennae 2) and we performed Real Time PCR with intronic primers for Ubx. Ubx expression is very low in antennae and in T1. In the T2, T3 and T4 appendages, Ubx is expressed at increasing level (Figure 27). This is similar to the

Ubx expression pattern during *Parhyale* embryogenesis (Pavlopoulos, Kontarakis et al. 2009).

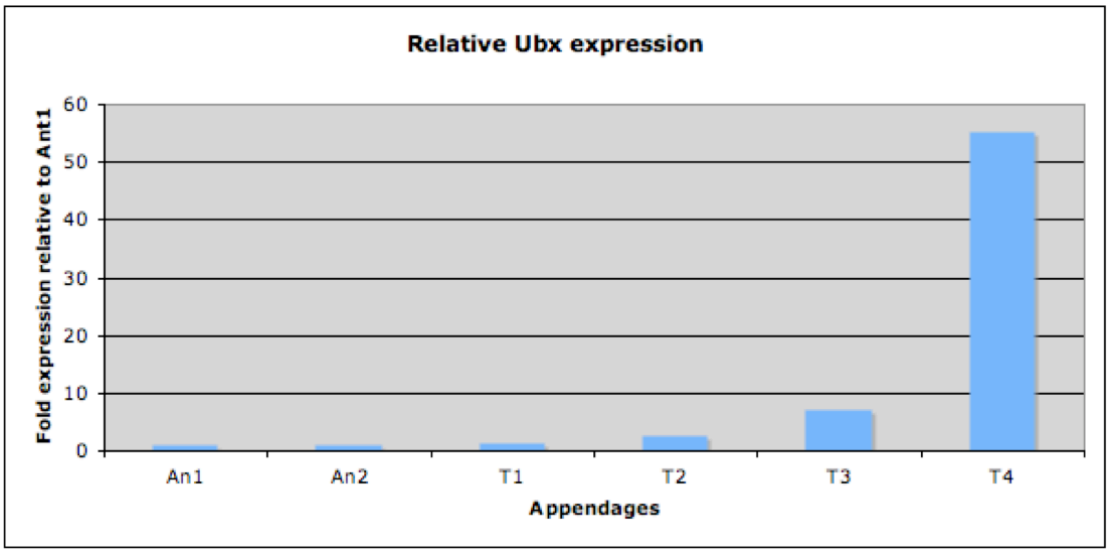


Figure 27: Ubx expression in adult appendages
Real-Time RT-PCR shows that Ubx is expressed in very low levels in cephalic appendages and maxillipeds (T1), in low levels in the second and third thoracic appendages and in higher levels in T4.

Having that in mind, we decided to misexpress Ubx during appendage regeneration. We used a line that was available in the lab that expressed *Parhyale Ph*Ubx1 under the *Parhyale* heat-shock promoter (Pavlopoulos, Kontarakis et al. 2009). The experimental procedure was the following: (a) we heat-shocked the animals at 37^{o}C for 1 hour, (b) immediately after heat-shock we amputated one of the first antennae and one of the third thoracic appendages, (c) we heat-shocked the animals 1 hour per day for four days after amputation. Subsequently, we let the animals regenerate and recorded the identity of the regenerates. 9 out of 47 animals were carrying homeotic transformations. 6 of them had partial transformations of the antenna to thoracic appendage and the other 3 were carrying partial transformation of the antenna to thoracic leg and of the third thoracic to walking leg (Figure 28).

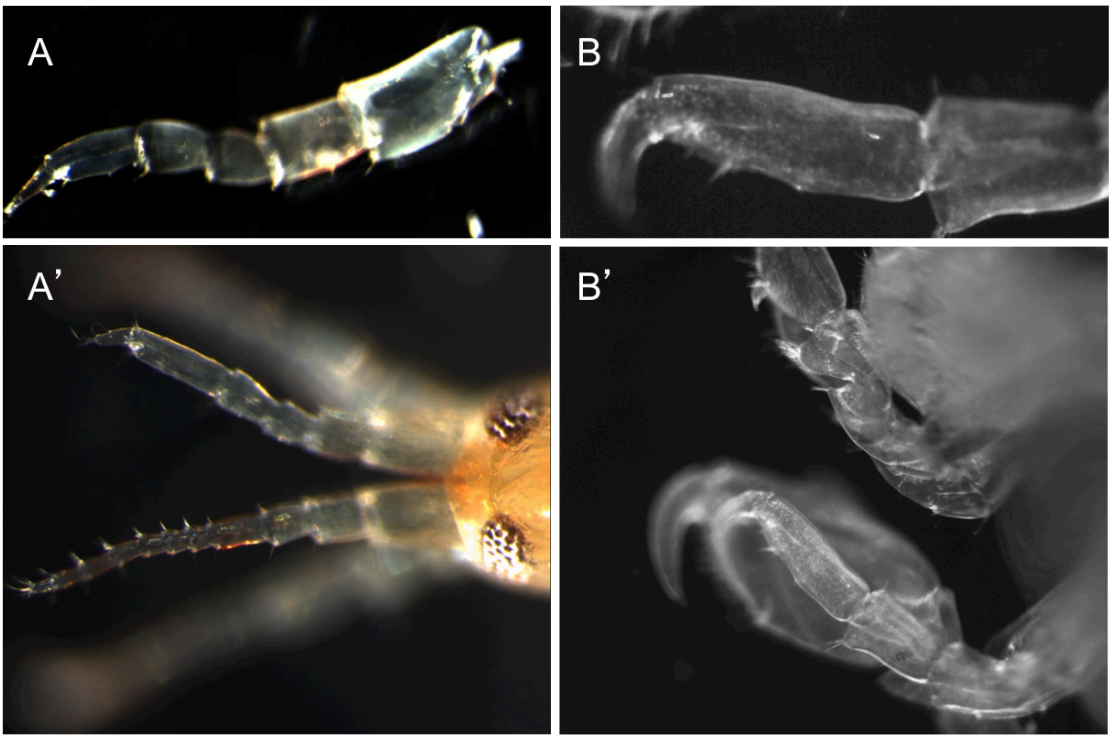


Figure 28: Ubx misexpression during regeneration causes homeotic transformations
Overexpression of Ubx during regeneration of antennae (A and A') and third thoracic appendages (B and B') causes partial homeotic transformations towards walking legs. The transformed appendages can be compared to the wild-type appendages of the other side of the animal which were not subjected to amputation and regeneration. These experiments were carried out on (non-mosaic) individuals from transgenic lines mis-expressing *Ph*Ubx1 under the control of the *Ph*HS element, following the heat shock regime shown in Figure 29.

We then wondered whether the transformation was temporary or permanent, so we let four of the animals to molt several times. The transformation persisted for many molts (up to four recorded molts). Subsequently, we amputated the transformed antennae either more proximally or more distally compared to the initial amputation and we let them regenerate without applying heat-shock. What we observed was that the persistence of the transformation was strictly dependent on the plane of amputation. Whenever the amputation was more proximal, the original identity was recovered. On the contrary, if the amputation was more distal, the transformation persisted. Figure 29 summarizes all the experiments.

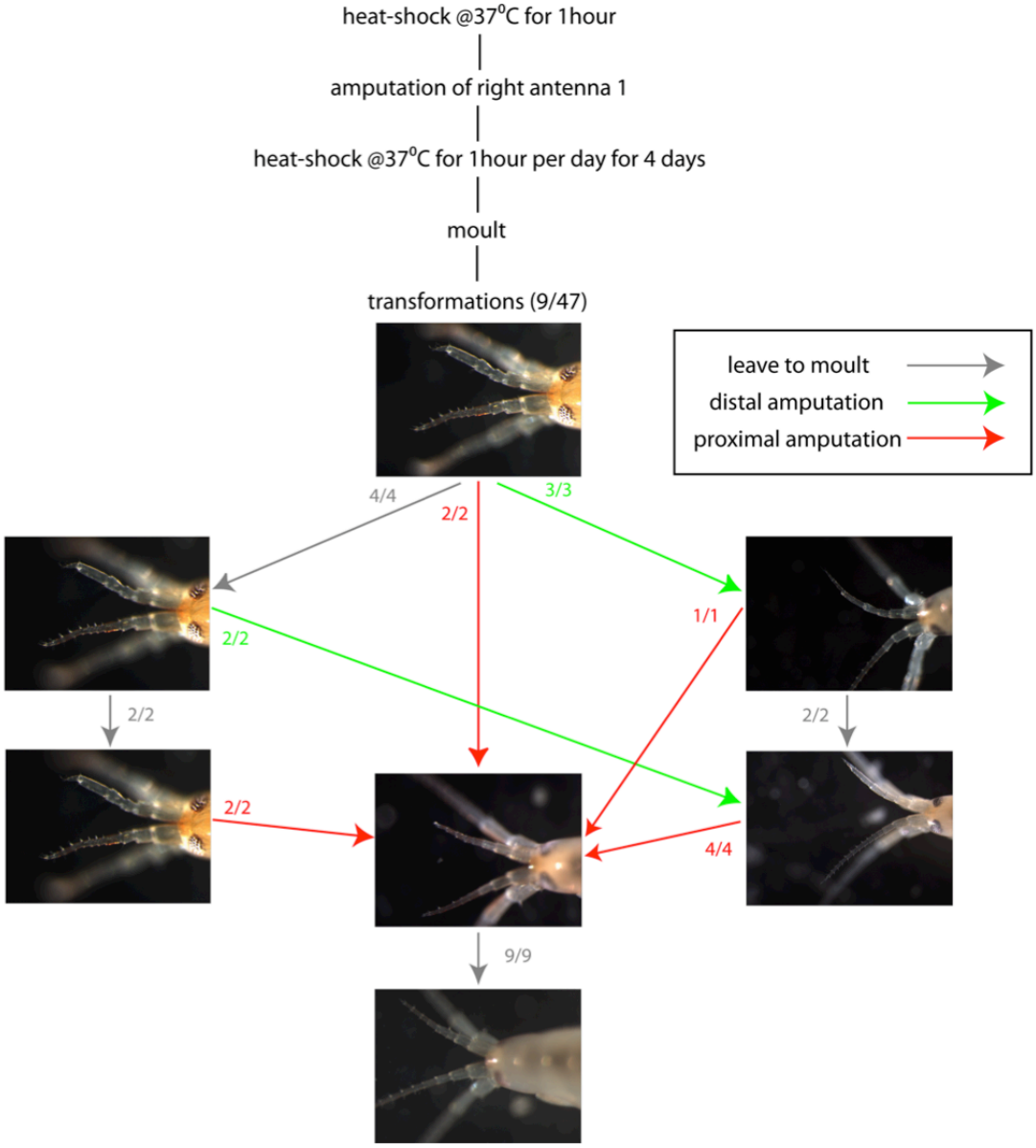


Figure 29: Experimental procedure and results of Ubx misexpression during antennal regeneration
Animals carrying the *Ph*HS-*Ph*Ubx1 construct were heat-shocked, and then their first antenna and third thoracic appendage were amputated. They were subjected to daily heat-shocks for the next 4 days. Phenotypes were recorded after the following moult. 9/47 antennae and 3/47 third thoracic appendages displayed partial homeotic transformations. Subsequently, the animals were either left to moult or their transformed antennae were amputated, either within or proximally to the transformed part of the antenna. Whenever the animals were left to moult or the antenna was re-amputated within the transformed part, the homeotic phenotype was maintained. Whenever the antenna was re-amputated proximally to the first amputation the homeotic phenotype was reversed..

If the amputation was on the same point, we recorded chimeric appendages like the one in Figure 30. However, these appendages after the next molt acquired the morphology of the original appendage prior to amputation.

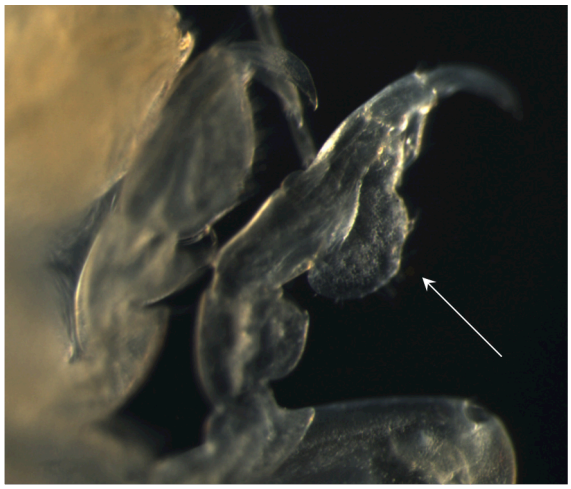


Figure 30: Appendage with chimaeric identity
A partially transformed T3 appendage (after initial amputation and subsequent *Ph*UbxI misexpression) was subjected to amputation at the same point as the amputation that lead to the transformation, without any further misexpression of *Ph*UbxI. After regeneration, it displays mixed characteristics of third thoracic and walking appendages, after being amputated.

The conclusions that can be drawn from these experiments are the following. First, it seems that Ubx plays a role during regeneration. This can be extracted from the fact that Ubx is still expressed in a segment-specific manner, which resembles the embryonic segmental expression and from the observation that the adult appendage identity is still susceptible to homeotic transformation. Moreover, homeotic transformations were observed only in regenerating appendages. No non-regenerating appendages were transformed. Furthermore, the cells that are at the plane of amputation seem to be the ones that determine the identity of the regenerated appendage, as shown by the secondary amputation experiments. Finally, the observation that the cells retain the "transformed" phenotype indicates that either the overexpression of Ubx changes the epigenetic regulation of the endogenous gene in the cells that participate in regeneration or that the phenotype, once established, does not need Ubx expression to be retained. This should be assessed with Real Time PCR. The number of transformed animals did not give us the opportunity to perform this experiment.

Collectively, these results represent the first direct evidence for the participation of a Hox gene in the segmental identity of the appendage both in embryonic development and in regeneration.

2.9 Plasticity of *Parhyale* regeneration progenitors

The consensus that derives from the regeneration studies described in 2.4 is that in vertebrates the cells cannot switch their lineages significantly. Moreover, there do not seem to exist totipotent cells that can give rise to every tissue. On the other hand planarians regenerate all their tissue types from a common pool of totipotent adult stem cells. Transdifferentiation, also, has been shown to occur in specific circumstances. In order to understand the evolutionary history of regenerative capacity, it is necessary to study other species, decipher whether they follow the planarian or the vertebrate paradigm and, potentially, identify other examples where transdifferentiation occurs. In this direction, I addressed this question in *Parhyale.*

Parhyale's stereotypic early cell lineage, which was described in Chapter 1, gives us the opportunity to create mosaic animals that express a transgene in specific lineages. In brief, the experimental procedure I followed to assess the regenerative potential of different lineages, consisted of the following steps. Initially, I injected the labeling construct in 2-cell, 4-cell and 8-cell stage embryos, marking different lineages. Subsequently, I screened late embryos for stable insertion of the transgene in specific lineages. I let the animals grow and when they reached adulthood, after confirming the lineage-specific fluorescence, I amputated several appendages. Upon regeneration, I scored the contribution of each lineage in the newly formed appendages.

Initially, I injected two different constructs that would cause heat-inducible expression of DsRed or EGFP, pMi(3xP3-DsRed;*Ph*HS-DsRed) and pMi(*Ph*HS-EGFP) respectively, together with *in vitro* transcribed transposase mRNA, in order to decide which of the two was more easily detectable. I concluded that EGFP was more appropriate for my analysis; therefore, I continued injections with this construct. Moreover, in a pilot experiment, I injected in 2-cell, 4-cell and 8-cell stage embryos, since the timing of the integration of the transgene in the genome is not yet clear. The embryos that were injected in the 8-cell stage generated mosaic animals with very restricted expression patterns. These animals were not useful, as I could not decipher the exact expression pattern of EGFP. Therefore, I continued by injecting in 2-cell and 4-cell stage embryos. Based on the blastomere, in which the construct has integrated, as well as the timing of the integration, different lineages may be marked. Close to the end of embryogenesis, in the eighth or ninth day, I heat-shocked the

survived embryos for one hour at 37°C. After 16-20 hours, I examined the embryos for EGFP expression to score for marked lineages. Collectively, I injected 4362 embryos, of which 1685 survived, 469 carried insertions of the construct and 79 were useful for my assay, i.e. they had a clear expression pattern in one or more of the 8-cell blastomere lineages. Out of the 79 animals that were used, 67 carried the insertion in a single 8-cell stage blastomere lineage, 9 in two of these lineages and 3 in four of them (Table 4 – displayed in the end of the chapter). Images of each labeled lineage in late embryonic stages can be seen in Figure 31.

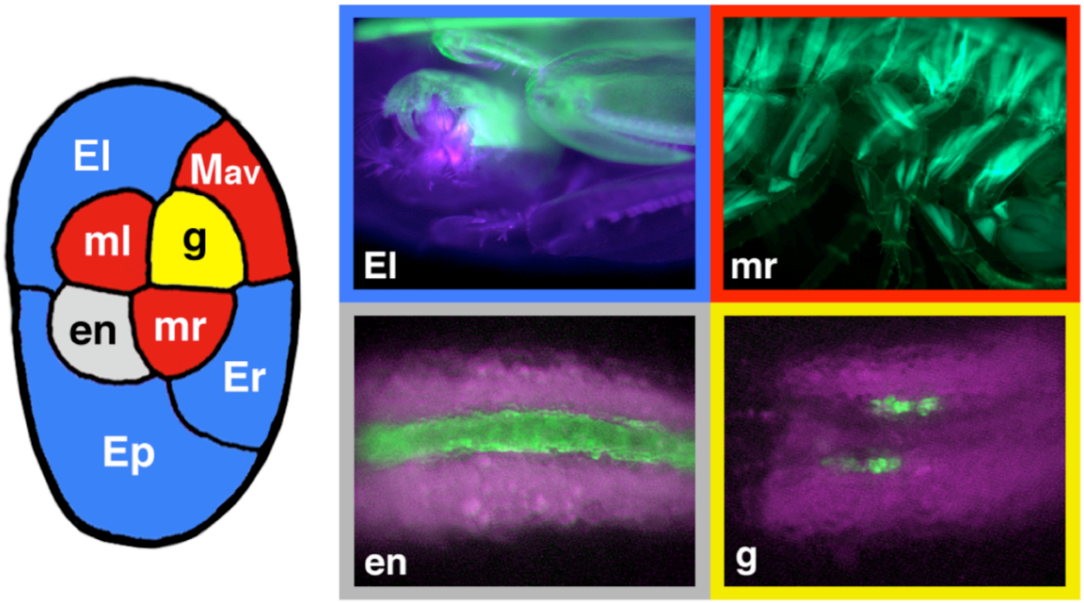


Figure 31: Generation of mosaic animals expressing EGFP in specific blastomere lineages
By injecting the pMi(*Ph*HS-EGFP) construct alongside with Minos transposase in early embryos, I generated mosaic animals that expressed EGFP under the control of the heat-inducible *PhHS* element within specific blastomere lineages. Representative animals are shown that express EGFP in the left ectoderm (El, ventral view) the right mesoderm (mr, lateral view), the endoderm (en, dorsal view) and the germ line (g, dorsal view).

Figure 32 illustrates the first three embryonic cell divisions as reported by Gerberding et al. There are two possible arrangements at the 4-cell and 8-cell stage (Mav and g can be either with ml and El or with mr and Er); hence, there exist two alternative lineage trees (Gerberding, Browne et al. 2002). Each of the tree branches corresponds to a single progenitor cell of the 2-cell, 4-cell and 8-cell stage embryo. The numbers on the branches indicate the number of mosaic animals that were labeled in this specific lineage.

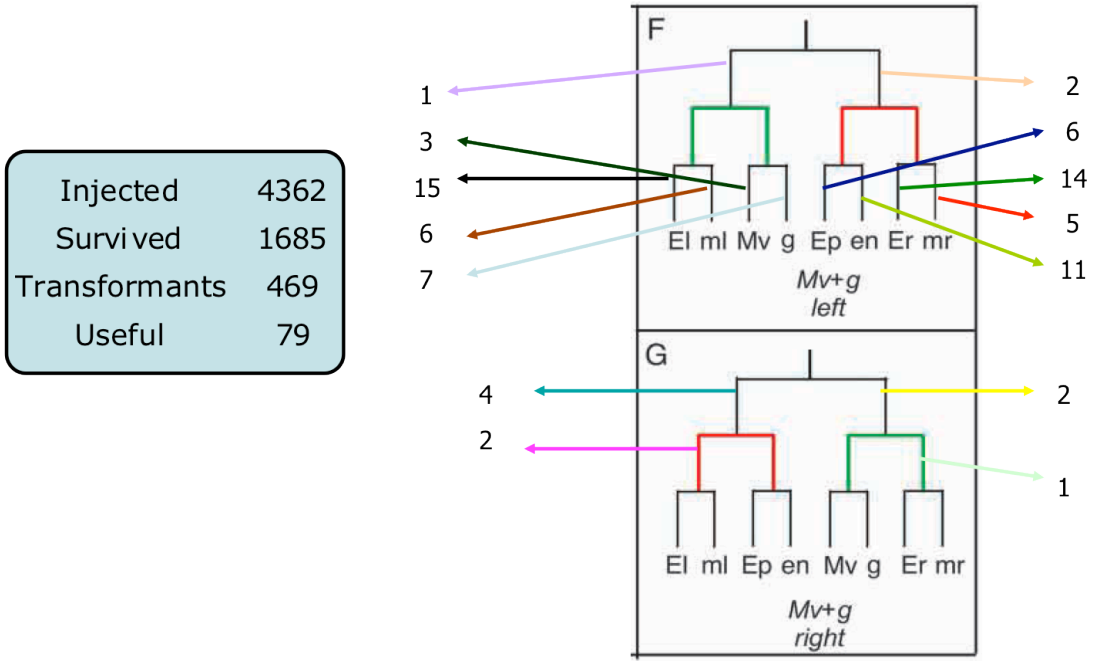


Figure 32: Summary of the blastomere lineages marked in 79 mosaic animals, used to assess the regenerative potential of each lineage
Out of 4362 injected embryos, 1685 were screened as late embryos and 469 were found to have EGFP expression restricted to one or more blastomere lineages. Out of these, 79 were found to be useful (animals that expressed EGFP in very restricted patterns were discarded). The lineage trees on the right depict the two alternative patterns of blastomere division leading to the 8-cell *Parhyale* embryo (taken from (Gerberding, Browne et al. 2002)). The numbers indicate how many animals had the transgene inserted in each cell, in 2-, 4- and 8-cell stage embryos.

The ones that were expressing EGFP and were considered useful (i.e. they had interpretable clone) were kept separately until adulthood. After two months, I heat-shocked them again and examined for EGFP expression to verify that the expression was consistent with that recorded in late embryogenesis (to discard animals with no integrated transposable elements). The animals that were injected as embryos in the El, Er, Ep, Mav, ml and mr blastomeres had exactly the same expression pattern, whereas I couldn't observe EGFP expression when the animals had the transgene integrated only in the en or only in g. In the case of en blastomere-injected animals, it has been proposed that it might be giving rise to extraembryonic tissue (N. Patel, personal communication) and this might be the reason why I couldn't see expression in the adults. In the case of g blastomere-injected animals, the expression was not visible either due to the autofluorescence of the underlying gut or due to the autofluorescence of the cuticle. I confirmed the integration of the construct in one of the g blastomere-injected animals by crossing it to a wild-type animal and getting transgenic progeny.

After confirming the tissues that were expressing EGFP, I amputated appendages (antennae or thoracic limbs T2-T8), which were either labeled or non-labeled, on the proximal half of the limb and let them regenerate. Up to 3 days after molting, I heat-shocked the animals as described earlier and examined the regenerates for EGFP expression. The complete report of marked germ layers, amputated appendages and the contributions of the marked germ layer in the regenerates, in all animals can be seen in Table 4 in the end of the chapter.

The contribution to anterior mesoderm was scored on antennae; contributions to left and right mesoderm were scored on thoracic limbs T2-T8; contributions to posterior ectoderm were scored on posterior limbs derived from the Ep lineage (when Ep was labelled) or on T8 limbs (when not labelled); contributions to left and right ectoderm were scored on anterior thoracic limbs and antennae. The regional subdivisions that I used to score regenerative contributions in the ectoderm and mesoderm are different, because they follow the body regions that are originally populated by each blastomere lineage; this was necessary in order to determine whether progenitor cells contribute to regeneration locally, at the site where they reside, or at a distance. Thus, the ectoderm was subdivided in left, right and posterior territories (populated by lineages El, Er and Ep) and the mesoderm was subdivided in left, right and anterior (populated by lineages ml, mr and Mav). Thus, observations from an amputated left antenna were scored as ‘left ectoderm’ and ‘anterior mesoderm’ (rather than ‘left mesoderm’). Conversely, observations from a posterior limb on the left side of the body were scored as ‘posterior ectoderm’ and ‘left mesoderm’.

Mosaics where more than one blastomere lineage was marked (see above) were not used to score the regenerative contributions of the marked lineages – as it would not have been possible to distinguish which lineage had made the contribution – but rather to test the absence of a regenerative contribution. For example, individuals with marked El and ml lineages allowed me to test the absence of regenerative contributions to the mesoderm of limbs on the right side of the body and of both antennae, and to the ectoderm of limbs on the right side of the body and at the posterior (on both sides).

I consistently observed that each marked lineage contributes to the regeneration of the tissues that derived from this lineage during embryonic development. For example,

the ectodermal lineage contributed to the regeneration of epidermis and neurons (Figure 33A). Likewise, the mesodermal lineage gave rise to muscles (Figure 33B). As seen in Figure 33, unamputated appendages are identical to the amputated and regenerated ones.

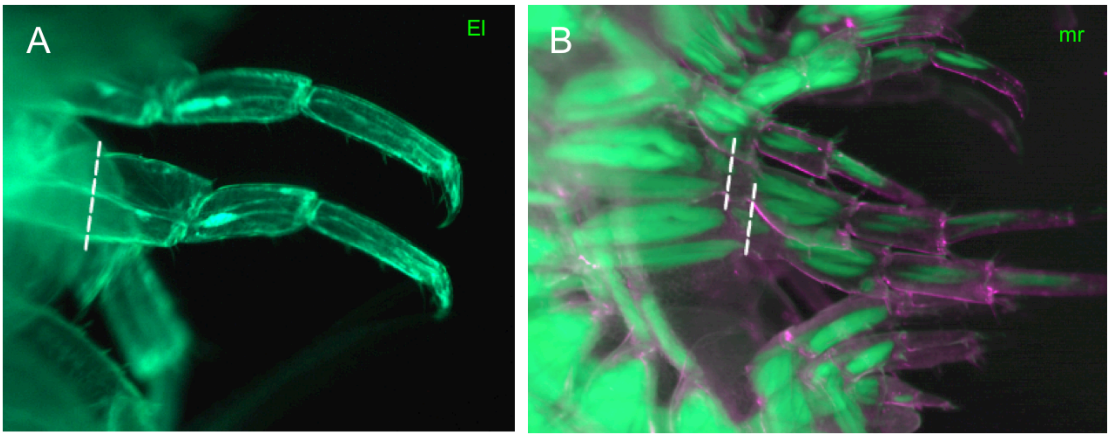


Figure 33: Ectodermal and mesodermal blastomere lineages contribute to the regeneration of limb ectoderm and mesoderm, respectively
After regeneration of amputated thoracic legs, ectodermal cells derived from the El lineage contribute to the regeneration of the epidermis and nerve cells (A), and mesodermal cells derived from the mr lineage participate in the regeneration of muscle (B). As a consequence, the regenerated appendage expresses EGFP in a pattern that is identical to that of non-amputated appendages. Planes of amputation are indicated by dashed lines. Magenta in (B) indicates reflected light.

In two limb amputations, I was not able to score the expected contribution to the regenerated tissues. In the first, marked ectodermal cells in the left side did not contribute to the regeneration of epidermis and neurons of the left appendage. In the second case, left mesodermal cells did not participate in muscle regeneration of a left side limb. This could be attributed to the mosaicism of the transgene integration and may be explained by the lack of transformation of some progenitors that led to the regeneration of unlabeled tissues.

Table 4 depicts the contributions of marked lineages (vertically) to the regeneration of each germ layer's tissues (horizontally).

Regenerated tissues		Ectoderm			Mesoderm		
		Left	Right	Posterior	Left	Right	Anterior
Marked lineages	El (n=19)	26/27	0/27	0/7	0/20	0/19	0/23
	Er (n=18)	0/25	21/21	0/10	0/19	0/18	0/21
	Ep (n=11)	0/18	0/21	10/10	0/14	0/20	0/21
	Ml (n=12)	0/6	0/9	0/8	10/11	0/11	0/10
	Mr (n=10)	0/13	0/7	0/9	0/14	11/11	0/8
	Mav (n=6)	0/7	0/6	0/8	0/11	0/10	12/12
	En (n=13)	0/21	0/38	0/22	0/25	0/25	0/34
	g (n=8)	0/17	0/21	0/16	0/18	0/14	0/21

Table 3: Regeneration progenitors are lineally-restricted and reside locally
Contribution of each of the lineages El, Er, Ep, ml, mr, Mav, en and g in the regeneration of ectodermal and mesodermal tissues of the regenerated appendage. The number of appendages in which each marked lineage participated in the regeneration of the respective tissues per number of appendages tested is indicated. Embryos that were marked in lineages El, Er and Ep, as well as ectodermal tissues are colored in blue, whereas ml, mr and Mav lineages, as well as mesodermal tissues are in red. n is the number of animals where each lineage was marked.

The outcome of this experiment is that regenerative progenitor cells have a developmental potential that is restricted with respect to germ layers. We have rigorously proven that ectodermal cells participate in the regeneration of ectodermal tissues only, while mesodermal cells regenerate only mesodermal tissues. Endoderm and germ line do not contribute to appendage regeneration. This situates *Parhyale* in the same category with vertebrates in terms of progenitor cell plasticity and, clearly, separates *Parhyale*'s regeneration process from what is observed in planarians. Whether the lineage-restricted progenitors are adult stem cells or dedifferentiated cells cannot be answered by this experiment and remains to be tested. This question will be partly addressed in Chapter 3.

A second conclusion that can be drawn from these experiments is the local contribution of each lineage, and, specifically, to the region that it originally populated. El and ml lineages contribute to the regeneration of the left side's ectoderm and mesoderm, whereas Er and mr regenerate only the right side's tissues. This local contribution is also true for Ep (regeneration of posterior ectoderm) and Mav (regeneration of antennal mesoderm). This implies that the progenitors are also regionally restricted and remain in the limb during regeneration.

Thus, within each limb, regeneration involves the interplay of at least two distinct types of progenitor cells – ectodermal and mesodermal – that have different origins and pre-determined (lineally-restricted) capacities.

a/a	Blastomere lineage	Specifically	Amputated	Contributions to					
				Mesoderm left	Mesoderm right	Mesoderm anterior	Ectoderm left	Ectoderm right	Ectoderm posterior
2	El	antennae, T1-T4	left T3	0/1			0/1		
3	El	antennae	left Ant2, left T7	0/1		0/1	2/2		
5	mr		right T7, rigth T8		2/2			0/1	0/1
6	El	antennae, T1-T3	left T2, left T3, left T6, right T2	0/3	0/1		2/2	0/1	0/1
9	Er, mr	antennae, T1-T4	left T2, left T3, left T7	0/3			0/3		
11	ml		left Ant2, left T5, right Ant2, right T5	1/1	0/1	0/2	0/2	0/2	
12	El	antennae, T1-T8	left T3, left T6, right T6	0/2	0/1		2/2	0/1	
14	El	antennae, T1-T6	left T3, right T3	0/1	0/1		1/1	0/1	
18	Er	antennae	left Ant1, right Ant1			0/2	0/1	1/1	
23	Er	antennae	left Ant2, right Ant2			0/2	0/1	1/1	
24	Er, mr, Mav, g	antennae, T1-T5	left T3, left T6, right T5, right T6	0/2			0/1		0/2
27	El, ml		left T4, right T4		0/1			0/1	
30	ml, mr		left T5, right T5				0/1	0/1	
31	Ep, ml	T5-T8	left T3, left T7, right T3, right T7		0/2		0/1	0/1	
32	El	antennae	left Ant2, right Ant2			0/2	1/1	0/1	
35	ml		left T7, right T7	1/1	0/1				0/2
36	El	antennae, T1-T3	left T2, right T2	0/1	0/1		1/1	0/1	
39	mr		left T4, right T4	0/1	1/1		0/1	0/1	
40	Er	antennae	left Ant2, right Ant2			0/2	0/1	1/1	
42	mr, Mav		left Ant1, left T8, right Ant1, right T8	0/1			0/1	0/1	0/2
43	Er	antennae, T1-T5	left T3, left T7, right T3, right T7	0/2	0/2		0/1	1/1	0/2
44	Er	antennae	left Ant1, right Ant1			0/2	0/1	1/1	

49	mr		left T8, right T8	0/1	1/1				0/2
51	ml		left T4, right T5	1/1	0/1		0/1	0/1	
52	El, ml	antennae, T1-T6	left T7, left T8, right T7, right T8		0/2				0/4
53	El, Ep	antennae, T1-T5	left Ant1, right Ant1, right T3, right T4		0/2	0/2		0/3	
54	ml, Mav		left T8, right T3, right T4, right T8		0/3		0/1	0/1	0/2
55	Er, mr, Ep, en	antennae, T1-T6	left Ant2, left T3, left T4, right Ant2	0/2		0/2	0/3		
56	El, ml, Ep, en		left Ant1, left Ant2, right Ant1, right Ant2			0/4		0/2	
58	Er, Ep		right T7, right T8		0/2				
59	Mav		left Ant1, left Ant2, left T3, left T5, left T8, right Ant1, right Ant2, right T3, right T5, right T8	0/3	0/3	4/4	0/4	0/4	0/2
428	mr		left Ant1, left T4, left T5, left T8, right Ant1, right T5, right T6, right T8	0/3	3/3	0/2	0/3	0/3	0/2
101	El	antennae	left Ant1, left T3, left T8, right Ant1, right T3, right T8			0/2	1/1	0/1	
102	El	antennae	left Ant1, right Ant1			0/2	1/1	0/1	
103	El	antennae, T1-T2	left T2, left T6, right Ant2, right T2	0/2	0/1	0/1	1/1	0/2	0/1
105	Er	antennae, T1-T4	left T3, left T6, right Ant2, right T3	0/2	0/1	0/1	0/1	2/2	0/1
106	Ep	T6-T8	left Ant1, left T3, left T7, left T8, right Ant1, right T3, right T7, right T8	0/3	0/3	0/2	0/2	0/2	4/4
108	Er	antennae, T1-T3	left Ant2, left T2, right T2, right T6	0/1	0/2	0/1	0/2	1/1	0/1

111	El	antennae, T1-T8	left Ant1, left T4, left T5, left T8, right Ant1, right T5, right T8	0/3	0/2	0/2	4/4	0/3	
112	Er	antennae, T1-T8	left Ant1, left T3, left T6, right Ant1, right T3, right T6	0/2	0/2	0/2	0/3	3/3	
113	Er	antennae, T1-T3	left T3, left T6, right T3	0/2	0/1		0/1	1/1	0/1
114	Ep	left T3-T8	left Ant2, left T2, right T2, right T5, right T6	0/1	0/3	0/1	0/2	0/3	
115	El	antennae, T1-T8	left T3, left T7, right T3, right T7	0/2	0/2		2/2	0/2	
116	Er	antennae, T1-T4	left Ant1, left T3, right Ant2, right T3, right T5. right T6, right T7	0/1	0/4	0/2	0/2	2/2	0/3
117	Er	antennae	right Ant2					1/1	
118	El	antennae, T1-T3	left Ant1, left T2, right T2, right T6	0/1	0/2	0/1	2/2	0/1	0/1
119	Er	antennae	left Ant1, right Ant1			0/2	0/1	1/1	
121	Er	antennae, T1-T8	left T3, left T7, right T3, right T4, right T7, right T8	0/2	0/4		0/2	4/4	
123	El	antennae, T1-T8	left Ant1, left T3, left T7, left T8, right Ant1, right T3, right T7, right T8	0/3	0/3	0/2	4/4	0/4	
124	Er	antennae	left Ant2, right Ant2			0/2	0/1	1/1	
129	Ep	left T6-T8	left Ant2, left T3, left T8, right Ant2, right T3, right T8	0/2	0/2	0/2	0/2	0/3	1/1
131	Ep	right T5-T8	left Ant2, left T3, left T8, right Ant2, right T3, right T8	0/2	0/2	0/2	0/3	0/2	1/1
134	Ep	left T6-T8	left Ant1, left T3, left T7, right Ant1, right Ant2, right T3	0/2	0/1	0/3	0/2	0/3	1/1
135	El	antennae	left Ant1, left Ant2, right Ant1, right Ant2			0/4	2/2	0/2	
137	Ep	right T4-T8 left T6-T8	left Ant1, left Ant2, left T2, left T8, right Ant1, right T2, right T4, right T5	0/2	0/3	0/3	0/3	0/2	3/3

201	en		left Ant1, left T3, left T8, right Ant1, right Ant2, right T3, right T8	0/2	0/2	0/3	0/2	0/3	0/2
202	en		left Ant1, left T3, left T6, left T8, right Ant1, right Ant2, right T3, right T8	0/3	0/2	0/3	0/3	0/3	0/2
203	en		left T3, left T8, right Ant1, right Ant2, right T3, right T8	0/2	0/2	0/2	0/1	0/3	0/2
204	en		left Ant1, left T3, left T8, right Ant1, right Ant2, right T3, right T8	0/2	0/2	0/3	0/2	0/3	0/2
205	en		left Ant1, left T3, left T8, right Ant1, right Ant2, right T3, right T7, right T8	0/2	0/3	0/3	0/2	0/4	0/2
206	en		left T3, left T8, right Ant1, right Ant2, right T3, right T7, right T8	0/2	0/3	0/2	0/1	0/4	0/2
207	en		left T3, left T8, right Ant1, right Ant2, right T3, right T8	0/2	0/2	0/2	0/1	0/3	0/2
208	en		left T3, left T8, right Ant1, right Ant2, right T3, right T8	0/2	0/2	0/2	0/1	0/3	0/2
209	en		left T3, left T8, right Ant1, right Ant2, right T3, right T4, right T8	0/2	0/3	0/2	0/1	0/4	0/2
210	en		left Ant1, left T3, left T8, right Ant1, right Ant2, right T3, right T8	0/2	0/2	0/3	0/2	0/3	0/2
211	en		left Ant1, left T3, left T8, right Ant1, right Ant2, right T3, right T8	0/2	0/2	0/3	0/2	0/3	0/2
301	g		left T3, left T8, right Ant1, right Ant2, right T3, right T4, right T8	0/3	0/2	0/2	0/2	0/3	0/2
302	g		left T3, left T4, left T8, right Ant1, right Ant2, right T3, right T8	0/3	0/2	0/2	0/2	0/3	0/2

303	g		left Ant1, left T3, left T8, right Ant1, right Ant2, right T3, right T8	0/2	0/2	0/3	0/2	0/3	0/2
304	g		left Ant1, left T3, left T8, right Ant1, right Ant2, right T3, right T8	0/2	0/2	0/3	0/2	0/3	0/2
305	g		left Ant1, left T3, left T8, right Ant1, right Ant2, right T3, right T8	0/2	0/2	0/3	0/2	0/3	0/2
306	g		left Ant1, left Ant2, left T3, left T8, right Ant1, right Ant2, right T3, right T8	0/2	0/2	0/4	0/3	0/3	0/2
307	g		left Ant1, left Ant2, left T3, left T8, right Ant1, right Ant2, right T3, right T8	0/2	0/2	0/4	0/3	0/3	0/2
425	ml		left Ant2, left T3, left T6, left T7, right Ant2	2/3		0/2			
430	ml		left T2, left T4	2/2					
431	ml		left Ant2, left T2, left T4, left T5, right Ant2	3/3		0/2			
432	Mav		left Ant1, left Ant2, left T3, left T5, left T6, right Ant1, right Ant2, right T3, right T6	0/3	0/2	4/4			
433	Mav		left Ant1, left Ant2, left T3, left T8, right Ant1, right Ant2, right T3, right T6, right T8	0/2	0/2	4/4			
443	mr		left Ant1, left Ant2, left T3, right Ant1, right Ant2, right T3, right T5, right T8	0/1	4/4	0/4			

Table 4: Inventory of the mosaic animals that were analyzed to investigate the contribution of each blastomere lineage to regenerated appendages.
The marked lineage ("Blastomere lineage"), the appendages that were amputated ("Amputated") and the contribution of the marked lineage to the regenerated appendages are indicated ("Contributions to"). The boundary between El or Er and Ep is variable within each animal. Therefore, in column "Specifically", I indicate the specific appendages that were marked, whenever El, Er or Ep lineages carried the transgene. In the columns "Contributions to", the number of appendages in which the marked lineage contributed to the regeneration of the respective tissue per number of appendages assayed is denoted.

Chapter 3

Satellite-like cells

Aside from the progenitors' regenerative potential (which cell types they are able to make), it is very interesting to know the progenitors' origin. Regeneration progenitor cells might come from an adult stem cell pool, whose job is to repair and/or regenerate tissues, or from differentiated cells, which when needed dedifferentiate and proliferate to generate new cells, participating in the restoration of the corresponding tissue. Of course, it might be the case that both adult stem cells and differentiated cells participate in regeneration as progenitors. A cell type, whose participation in regeneration has gathered a lot of attention lately, is the muscle satellite cells.

The participation of satellite cells in muscle formation during regeneration was first shown in *Xenopus* tadpoles. It was shown that the skeletal muscles of the tail were regenerated from the proliferation of muscle satellite cells. In particular, by grafting GFP expressing cells from the medial presomitic mesoderm in the neurula stage of *Xenopus* embryos, mosaic animals were produced that expressed GFP in the muscle fibers and not in the satellite cells. After tail regeneration, no GFP was observed in the muscles. This was reversed if later stage presomitic mesoderm was grafted, which produced GFP expressing satellite cells and muscle fibers (Gargioli and Slack 2004). Similarly to the observations in the *Xenopus* tadpole, it was proposed that satellite cells were responsible for muscle regeneration in the amphioxus (Somorjai, Somorjai et al. 2012) and the salamander (Morrison, Loof et al. 2006; Morrison, Borg et al. 2010). In both cases, which will be described in greater detail in the next section, the participation of dedifferentiated muscle fibers can not be excluded.

In contrary, during zebrafish heart regeneration, no participation of stem cells was detected in the restoration of cardiac muscles. Using Cre/lox technology, two different labs were able to mark differentiated cardiomyocytes irreversibly and to monitor their behavior after heart transection. They identified that the labeled cells generated all the new cardiomyocytes (Jopling, Sleep et al. 2010; Kikuchi, Holdway et al. 2010). Moreover, the re-expression of an embryonic cardiogenesis marker, GATA-4, indicates the dedifferentiation of cardiomyocytes to embryonic progenitors, before proliferation (Kikuchi, Holdway et al. 2010).

In this Chapter, I will focus on satellite cells, introducing their role in development, repair and regeneration, since their participation in regeneration is relevant to my research. Afterwards, I will present data that I obtained following a candidate cell approach that suggest that *Parhyale*'s muscle progenitors during appendage regeneration are equivalent to the vertebrates' satellite cells.

Introduction

3.1 Muscle satellite cells

General features

Muscle satellite cells have been shown to be fundamental for muscle regeneration in some chordates. They have been proven to be involved in muscle homeostasis, repair and regeneration (Gargioli and Slack 2004; Sacco, Doyonnas et al. 2008; Morrison, Borg et al. 2010; Lepper, Partridge et al. 2011; Sambasivan, Yao et al. 2011; Wang and Rudnicki 2012). In order to distinguish satellite cells' role in the maintenance of functional muscles, and their role in regeneration, which is triggered by amputation, I will refer to the first situation as muscle repair and to the second as muscle regeneration. In the literature these two terms are sometimes confused. The information presented in this Introduction comes from studies in chordates, where satellite cells have been identified and studied

Satellite cells were initially detected fifty years ago, when Mauro saw through the electron microscope a small, mononucleated cell, between the muscle fiber and the basal lamina, which he named satellite cell (Mauro 1961). Surprisingly, he hypothesized that this might be the cell responsible for the replenishment of muscle fibers, without having any evidence for this at the time He was right. The position of a satellite cell relative to the muscle fiber can be seen in Figure 34A.

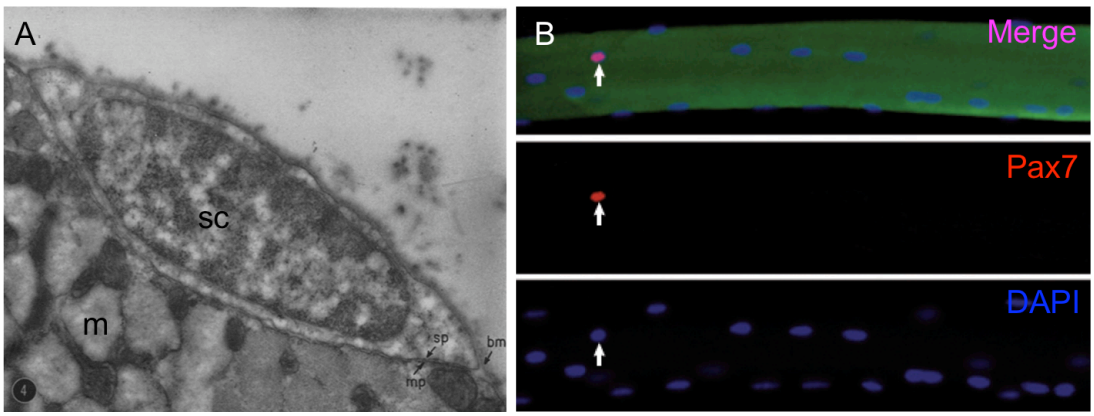


Figure 34: Vertebrate muscle satellite cells
(A) Satellite cells (sc) are tightly attached to the muscle fibers (m) and lie under the same basal membrane (bm; image taken from (Mauro 1961)). (B) The main molecular marker used to identify muscle satellite cells in vertebrates is the transcription factor Pax7 (Seale, Sabourin et al. 2000), which is expressed by muscle satellite cells but not by differentiated muscle cells (image taken from (Wang and Rudnicki 2012)).

The main molecular marker for satellite cells is the paired box transcription factor Pax7 (Seale, Sabourin et al. 2000), which characterizes the quiescent state of satellite cells (Figure 34B). Pax7 contains a paired box, a homeobox and a transactivation domain. Pax7 has been shown to be involved in central nervous system development (Kawakami, Kimura-Kawakami et al. 1997), apart from its participation in muscle progenitors. Alternative splicing of the Pax7 mRNA has been held responsible for the specification of neurogenic versus myogenic fates (Ziman and Kay 1998). Pax7, despite being expressed in the whole life of satellite cells, has been proven to be necessary only during the early stages of postnatal growth and not during repair in adulthood (Lepper, Conway et al. 2009). Once activated, satellite cells express other markers, such as MyoD and Myf5, before expressing the terminal differentiation marker myogenin (Charge and Rudnicki 2004).

Although the satellite stem cell population seems to have similar properties, it is heterogeneous. Their heterogeneity is exhibited in a variety of ways. Markers, such as CD34 and Myf5, are expressed in subpopulations of satellite cells (Beauchamp, Heslop et al. 2000). Moreover, the levels of Pax7 expression vary in satellite cells; different levels of Pax7 are associated with the capability of these cells to differentiate (low levels) or to self-renew (high levels) (Rocheteau, Gayraud-Morel et al. 2012). The heterogeneity of satellite cells, with respect to their ability for self-renewal and commitment, had also been shown earlier in mice (Kuang, Kuroda et al. 2007). Finally, the proliferative history of satellite cells is considered to be heterogeneous (Brack and Rando 2012).

As stated earlier, regeneration capacity often declines with age. However, the age of satellite cells does not seem to play a role in their proliferative capacity. Transplantation of aged satellite cells showed that they were as effective as their younger counterparts (Collins, Zammit et al. 2007). Instead, their depletion plays an important role in age-associated impairment of regeneration (Collins, Zammit et al. 2007). Extrinsic signals are responsible for that, as highlighted by a study that achieved the restoration of the regenerative capacity of old satellite cells using parabiotically paired old and young animals achieving (Conboy, Conboy et al. 2005).

Satellite cells and muscle repair

As stated earlier, under normal conditions, satellite cells are mitotically inactive and reside close to the muscle fibers, underneath the basal lamina. Upon injury, the satellite cells step out of the basal lamina, enter the cell cycle and proliferate; some of their descendants renew the satellite cell pool and others differentiate into myoblasts. The triggers that activate this process are not yet known. It is thought that the disruption of muscle fibers might lead to the destruction of the satellite stem cell niche and to the loss of inhibitory signals that prevent proliferation and differentiation. Mechanical signals due to degeneration of the muscles have been shown to participate in the activation of satellite cells (Pisconti, Brunelli et al. 2006; Wozniak and Anderson 2007).

After the satellite cells have proliferated, and before they differentiate into myoblasts, they surround the degenerated muscles (Brack and Rando 2012). Later, the myoblasts divide, stop expressing Pax7, express myogenin and fuse to form myofibers. A schematic representation of the procedure alongside with the relevant molecular markers is shown in Figure 35A.

Satellite cells are necessary both for muscle stem cell maintenance and for muscle repair. They repopulate the muscle stem cell pool and repair muscle fibers, as shown by Cre/lox lineage tracing studies (Lepper, Conway et al. 2009; Shea, Xiang et al. 2010). Moreover, ablation of adult Pax7-positive cells has demonstrated their necessity in these processes (Lepper, Partridge et al. 2011; McCarthy, Mula et al. 2011; Murphy, Lawson et al. 2011; Sambasivan, Yao et al. 2011).

Several other cells participate in the regulation of quiescence and proliferation of satellite cells (Figure 35B). Signals from damaged myofibers, cytokine-secreting macrophages, and differentiation-preventing signals from fibroblasts are all involved in this process (Wang and Rudnicki 2012).

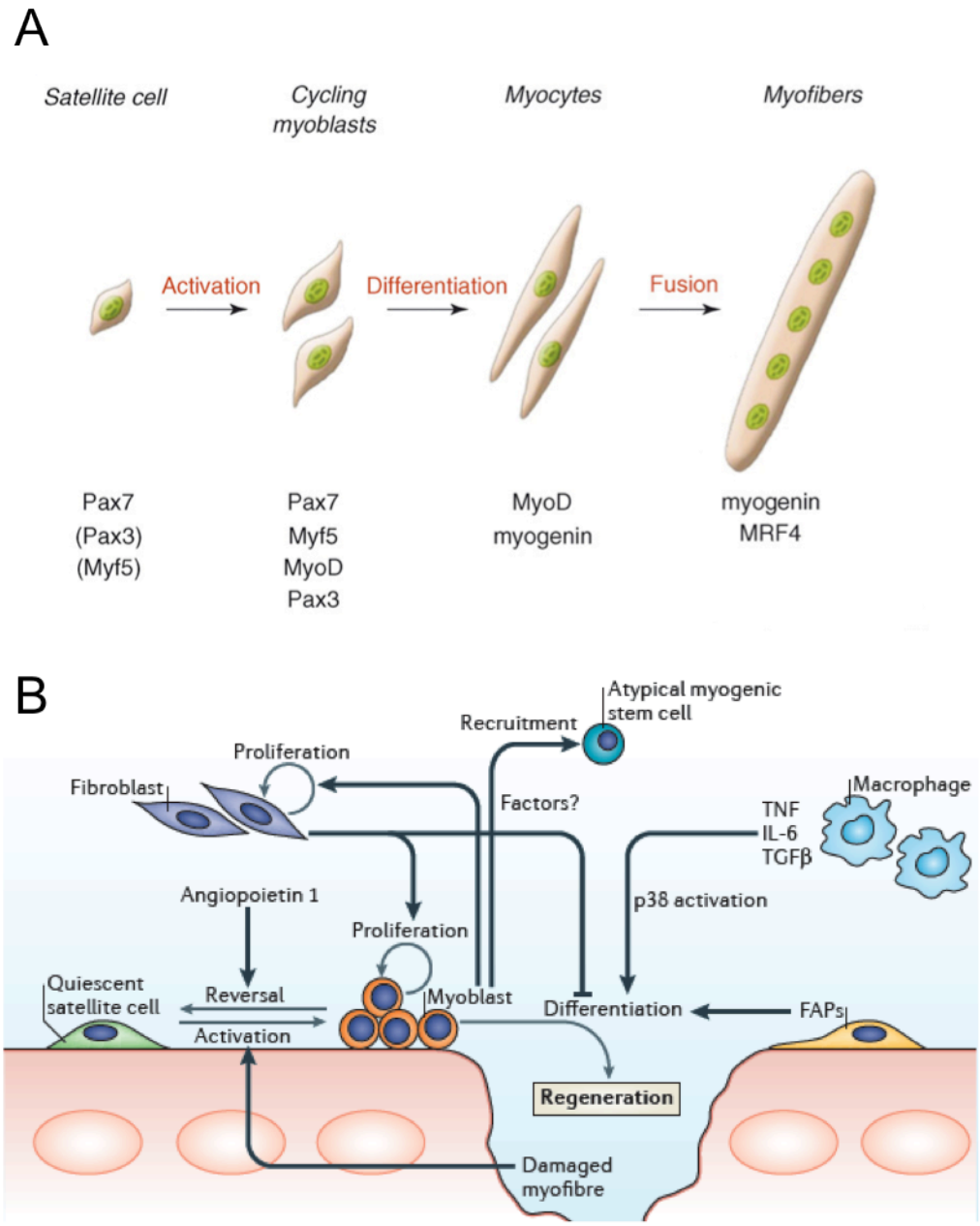


Figure 35: Satellite cell activation and differentiation in vertebrates
(A) During muscle maintenance or in response to injury, muscle satellite cells are activated and differentiate into myocytes. Different molecular markers characterize each stage in this process. Upon terminal differentiation, the myocytes fuse and form myofibers. Illustration taken from (Le Grand and Rudnicki 2007). (B) Several cell types have been shown to participate in satellite cell differentiation, by providing the appropriate cues for their activation. Illustration taken from (Wang and Rudnicki 2012).

Finally, it is interesting to note that the relationship between adult satellite cells and embryonic muscle progenitors is still under debate. The two populations show both differences (e.g. Pax3 expression (Williams and Ordahl 1994)) and similarities (e.g.

Pax7 and myogenic factor expression) (Fan, Li et al. 2012; Wang and Rudnicki 2012).

Satellite cells during limb or tail regeneration

The participation of satellite cells during muscle regeneration differs among different chordates.

Xenopus tadpole tail regeneration was the first case where satellite cells were proposed to participate in muscle regeneration (Gargioli and Slack 2004). It was later also shown that Pax7 plays an important role in muscle regeneration, by controlling the self-renewal of satellite cells. By overexpression of an antagonist of Pax7, muscle regeneration can be prevented following successive amputations. The fact that the initial amputation was successfully regenerated suggests that Pax7 does not have a direct role in muscle fiber formation but in maintenance of the satellite stem cell pool (Chen, Lin et al. 2006).

Within salamanders, muscle regeneration seems to involve different cell populations in different species. In newts, Pax7-expressing satellite cells have been shown to exist and to be activated upon limb amputation (Morrison, Loof et al. 2006; Morrison, Borg et al. 2010). However, their participation in muscle regeneration has been recently questioned. Instead, it has been shown that myofiber dedifferentiation plays an important role in muscle regeneration (Sandoval-Guzman, Wang et al. 2013). Contrary to the newt, in axolotls, Pax7-expressing cells are the main contributors to the newly formed muscle upon limb amputation (Sandoval-Guzman, Wang et al. 2013).

The European amphioxus *Branchiostoma lanceolatum* is able to regenerate anterior and posterior structures, including neural tube, notochord, fin, and muscle. Muscle regeneration proceeds with the proliferation of Pax3/7-positive satellite cells (in invertebrates, Pax3/7 is the homolog of Pax3 and Pax7) and their participation in the formation of the blastema. Their extent of activation is dependent on age, which is, in turn, associated with a decline of regenerative capacity (Somorjai, Somorjai et al. 2012). The presence and similar roles of satellite cells in amphioxus and vertebrates places the origin of satellite cells at least as late as the common ancestor of chordates.

Results - Discussion

3.2 Identification of satellite-like cells in *Parhyale*

My previous results on the lineage-restriction of the regeneration progenitors cells in *Parhyale* indicate that the progenitor cells of muscle fibers lie within the mesodermal lineage, locally in the limb (see Chapter 2). In order to identify them, we need to know what types of cells exist in the appendages that belong to the mesoderm. These types could then be studied to identify those that contributed to the mesoderm progenitor pool.

As a starting point, I studied transgenic animals that express DsRed under the control of the *Ph*MS regulatory element (Pavlopoulos and Averof 2005), which was known to be active in the muscle fibers. The PhMS regulatory element carries multiple putative binding sites for myogenic factors like MyoD. MyoD is a myogenic regulatory factor that specifies mesodermal cells to follow the skeletal muscle lineage. I constructed a Minos vector that carried EGFP under the control of the *Ph*HS element and DsRed under *Ph*MS element. I injected this construct in four- and eight-cell stage embryos, with the intention to target the mesodermal lineage. This way, I created mosaic animals (as described in Chapter 2) that express heat-shock inducible EGFP in the whole mesoderm and DsRed in the differentiated muscles. The rationale of this experiment was to identify non-muscle cells of the mesoderm, which would express EGFP but not DsRed.

I made two interesting observations in this experiment. The first observation was that the *Ph*MS-DsRed construct, which was lying upstream of the *Ph*HS-EGFP, was preventing the expression of EGFP even under heat-shock conditions. 4 out of 5 mosaics I generated did not express EGFP after heat-shock in the cells where *Ph*MS was active. I believe this is due to the ineffectiveness of the SV40 polyadenylation signal to terminate transcription that leads to read-through and transcriptional interference of the *Ph*HS-EGFP construct that follows. Only in one of my mosaics, I was able to observe the expression of both EGFP and DsRed. In this animal, the *Ph*MS-DsRed construct was active in all mesodermal cells that I could observe. This has two possible explanations. It could mean that *Parhyale* mesoderm consists solely of differentiated muscle fibers, which would mean that the muscle regenerative

progenitor cells are the muscle fibers themselves. Alternatively, it could mean that *Ph*MS is expressed in all limb mesodermal cells.

To distinguish between the two cases, I decided to look more carefully at the expression pattern driven by the *Ph*MS element. I fixed appendages from hatchlings of the *Ph*MS-DsRed line and I scanned them in the confocal microscope at single-cell resolution. Looking in detail, I was able to identify the presence of big circular cells interspersed between the muscle fibers (Figure 36). They were of similar size relative to each other, with a diameter of approximately 5-10um. There were one or two such cells per bundle of muscle fibers, closely associated to them. They could be found, as indicated by the arrows in Figure 36, either between bundles of muscle-fibers, where the two bundles met, or along these muscle-fibers. The location of these cells reminded me of the vertebrate satellite cells.

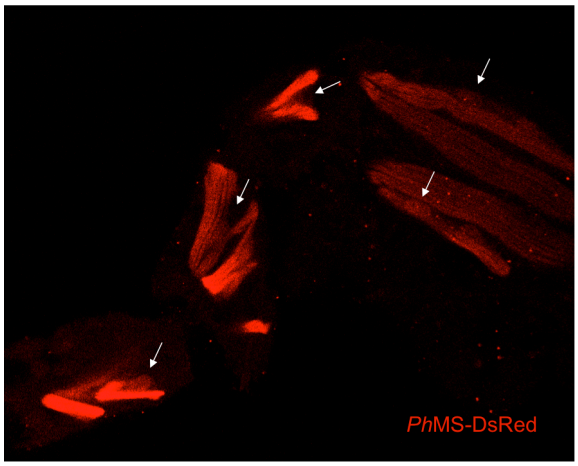


Figure 36: DsRed expression in *Parhyale* legs under the control of PhMS
DsRed expressed under the control of the *PhMS* element (Pavlopoulos and Averof 2005) is observed not only in differentiated muscles but also in small, circular cells positioned in proximity to the muscles. These cells are found between or along muscle bundles (arrows).

To see whether these muscle-associated cells express similar molecular markers as the vertebrate satellite cells, I studied Pax3/7 expression in *Parhyale* limbs. As mentioned in the introductory part of this Chapter, the key molecular marker for satellite cells is Pax7. Pax7 belongs to Pax3/7 family of paired box transcription factors. The duplication that separated Pax3 and Pax7 happened in the vertebrate lineage. Amphioxus has one member of the Pax3/7 family, *Drosophila* has three (*paired, gooseberry* and *gooseberry-neuro*), and *Parhyale* has three members (*pax3/7-1, pax3/7-2* and *pax3/7-3*) (Noll 1993; Somorjai, Somorjai et al. 2012) (Parchem 2008).

Fortunately, two cross-reactive monoclonal antibodies against the Pax3/7 family have been raised by the Patel lab, DP311 and DP312; these recognize members of the Pax3/7 family in diverse animals (Davis, D'Alessio et al. 2005) (Somorjai, Somorjai et al. 2012). Moreover, the antibodies were previously shown to recognize one of the members of the *Parhyale* Pax3/7 family, *Ph*Pax3/7-1 (Vargas-Vila, Hannibal et al. 2010). In *Drosophila*, DP311 recognizes *paired*, *gooseberry*, *gooseberry-neuro*, *aristalless*, *repo* and *Rx*. DP312, on the other hand, recognizes *paired*, *gooseberry*, *gooseberry-neuro* and *Rx* (Davis, D'Alessio et al. 2005). In my pilot experiments, DP311 and DP312 showed the same expression pattern in *Parhyale limbs*; DP311 was used in the subsequent experiments.

First, I performed antibody staining in late embryos, following the removal of the external embryonic membranes. In the latest embryos, the musculature of the limbs is formed. I wanted to see whether the muscle-associated cells expressed Pax3/7. I performed antibody staining with the DP311 antibody and an HRP-conjugated secondary antibody in late embryos carrying *Ph*MS-DsRed. As shown in Figure 37, I was able to identify Pax3/7 expressing cells in the vicinity of all muscle-fiber bundles in the podomeres and appendages I recorded. Moreover, these cells were in the same location as the ones I had observed previously, they were of the same size and there were one or two cells per muscle bundle, as described before.

To make sure that the antibodies were indeed recognizing Pax3/7 expression, I performed in situ hybridization with a probe for *Ph*Pax3/7-2 (Parchem 2008), which based on analyses of a *Parhyale* limb-specific transcriptome was most probably the *Ph*Pax3/7 family member expressed in the limbs. Figure 38 shows collective stainings with the two antibodies and the probe for *Ph*Pax3/7-2, confirming that DP311 and DP312 stain the same cells as *Ph*Pax3/7-2 in the legs, based on these cells' relative position to the muscle. During late embryonic stages staining becomes variable due to the cuticle preventing probe penetration. In this case, an AP-conjugated secondary antibody was used.

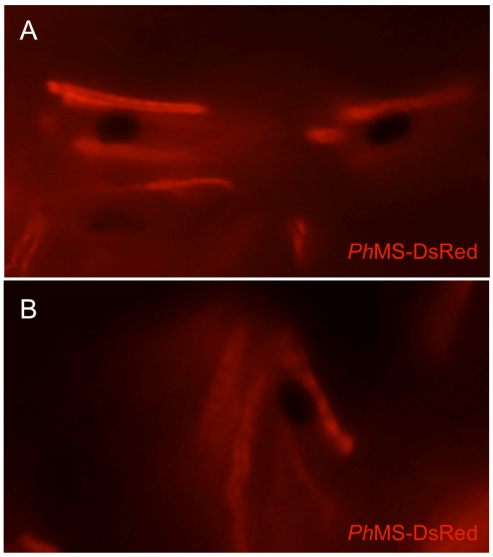


Figure 37: Pax3/7-expressing cells are associated with muscle fibers in *Parhyale* legs
Pax3/7-expressing cells, labelled with the DP311 antibody (black), are associated with muscle fibers (stained with phalloidin; red) in *Parhyale* thoracic legs.

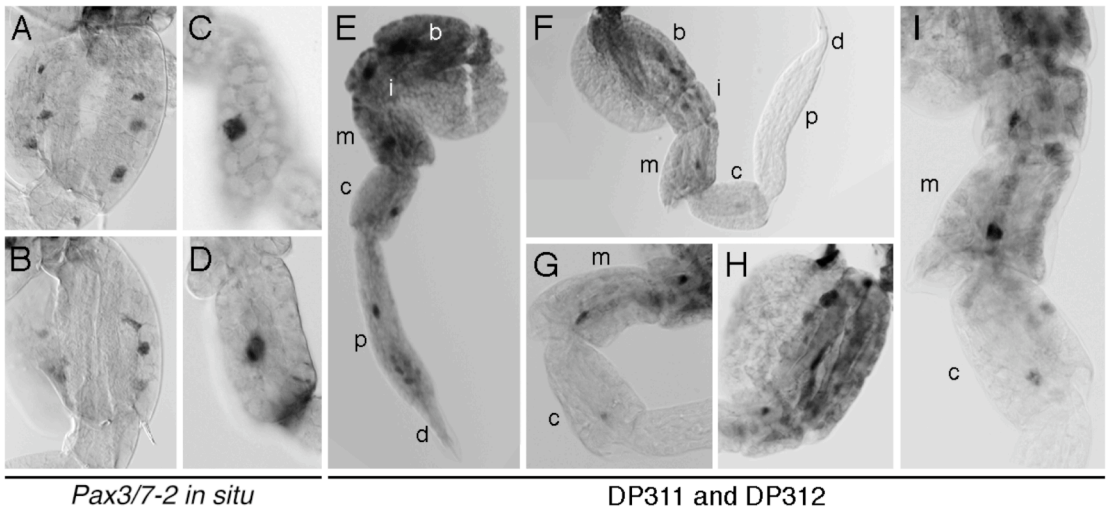


Figure 38: *In situ* hybridization shows that the muscle-associated cells, which are recognized by Pax3/7 antibodies DP311 and DP312, express *Ph*Pax3/7-2
(A-D) By *in situ* hybridization with a probe for *Ph*Pax3/7-2 in late *Parhyale* embryos, I identified several Pax3/7-expressing cells in the basis of thoracic appendages (A, B) and 1-2 cells in the ischium (A), the merus (C) and the carpus (D). (E-I) Antibody staining in late *Parhyale* embryos with antibodies against Pax3/7, DP311 and DP312, shows the same expression pattern in the basis (F, H), the ischium (E, F, H, I), the merus (E, F, G, I) and the carpus (E, G, I) and, also, shows occasional expression in the propodus (E). Variabilities in stainings are due to antibody and probe penetration problems that arise in late embryos due to the exoskeleton. The number of Pax3/7-positive cells increases with age and may vary among thoracic appendages. Leg podomeres: b, basis; i, ischium; m, merus; c, carpus; p, propodus; d, dactylus.

To better describe these cells, I combined antibody staining for Pax3/7 and DsRed, with phalloidin staining, in embryos from the *Ph*MS-DsRed line. Phalloidin recognizes and binds to the polymerized actin (F-actin), marking cells that are rich in polymerized actin, especially differentiated muscle fibers. In Figure 39A, differentiated muscles are in green (phalloidin), *Ph*MS-DsRed-expressing cells in red (DsRed) and Pax3/7-expressing cells in blue. These cells seem to be mesodermal, non-muscle fiber, Pax3/7 expressing cells. Moreover, these cells seem to have very little cytoplasm (as is described for vertebrate satellite cells); Pax3/7 is a transcription factor, most likely found in the nucleus, whereas DsRed is cytoplasmic.

I also wanted to verify the presence of these cells in adult limbs. Unfortunately, the exoskeleton presents an important obstacle to antibody penetration in adults. Therefore, I had to section the animals. Figure 39B depicts a transverse cryosection of an adult wild-type appendage (more than six months old). Satellite-like cells could be observed between muscle-fiber bundles, as phalloidin-negative and Pax3/7-positive cells. Satellite-like cells have a similar size to those observed in embryos (the muscle-fiber bundles are significantly larger in adult limbs).

In order to unambiguously prove that these cells are mesodermal I injected a construct that drives the expression of both nuclear EGFP and cytoplasmic Tomato under the *Ph*HS regulatory element (pMi(3xP3-EGFP;HS-lyntdTomato.2A.H2BEGFP)) (Kabrani 2012) in 8-cell stage embryos to generate mosaic animals that have a marked mesoderm. In five of these mosaic animals, I performed Pax3/7 antibody staining and showed that the Pax3/7-expressing muscle-associated cells express the mesodermal marker (7 out of 7 cells scored in late embryos) (Figure 39C). Collectively, these observations suggest that these cells have very similar characteristics to the vertebrate satellite cells.

From these stainings, I could confirm the consistent presence of a Pax3/7 positive, mesodermal, non-muscle-fiber, circular cell with little cytoplasm and a 5-10 um cell diameter in the vicinity of muscle fibers. From now on, I will call these cells satellite-like cells (SLCs).

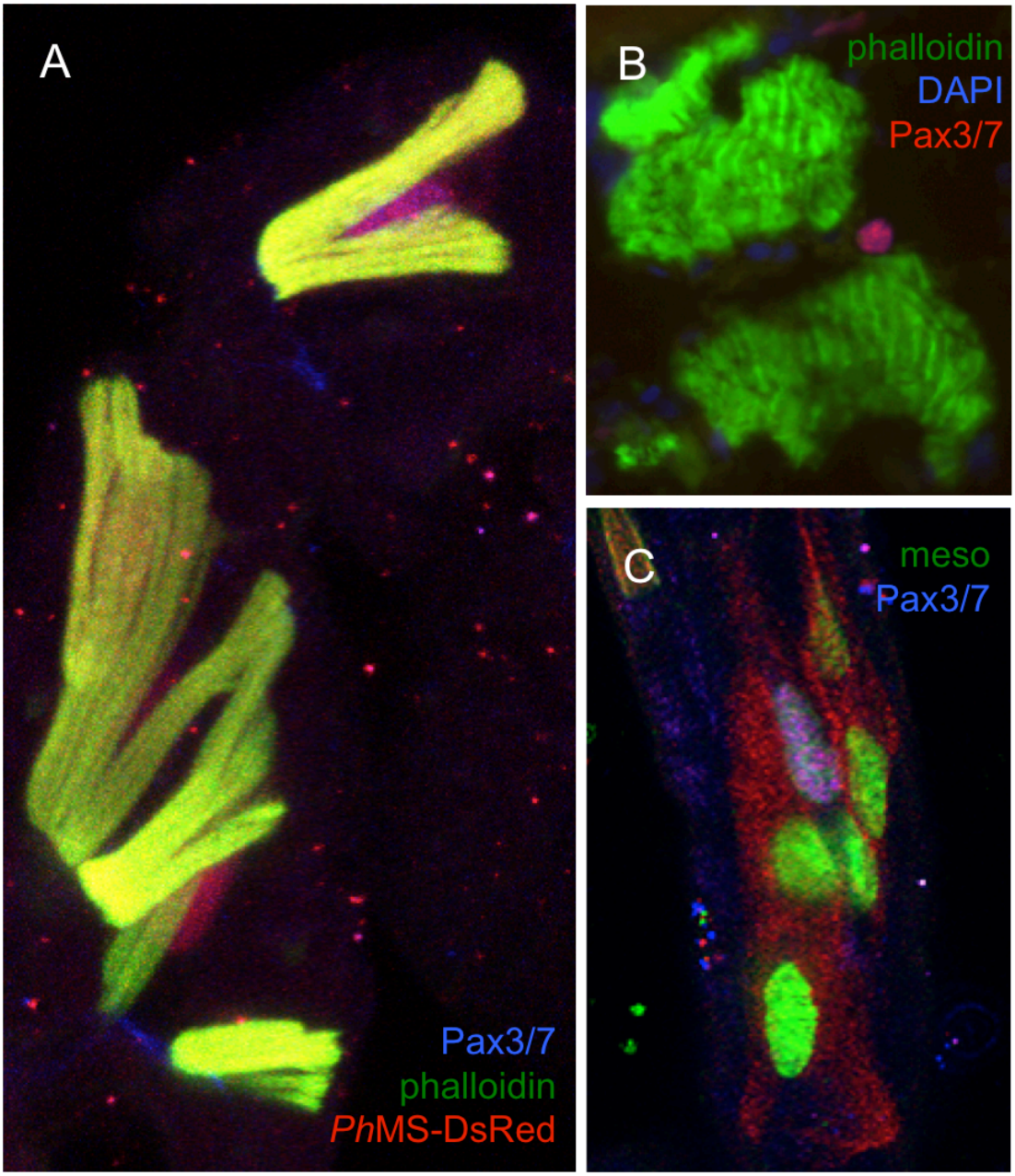


Figure 39: Satellite-like cells in *Parhyale*
(A) Double antibody staining against DsRed (red) and Pax3/7 (blue) combined with phalloidin staining of the ischium and merus of a thoracic appendage of a hatchling from the *Ph*MS-DsRed line. The aforementioned circular cells, which express the PhMS-DsRed reporter (red) and are tightly associated to the muscles, also express Pax3/7 (blue) and do not stain with phalloidin (green) in *Parhyale* hatchlings. (B) The same is true in adult animals, as can be seen in an antibody staining for Pax3/7 (red), combined with phalloidin (green) and DAPI (blue) staining, of transverse sections of adult *Parhyale* appendages. (C) To prove that Pax3/7-expressing cells are mesodermal, I generated mosaic animals, which carry tdTomato and nuclear EGFP under the heat-shock promoter in the mesoderm, by injecting the Minos construct pMi(3xP3-EGFP;HS-lyntdTomato.2A.H2BEGFP) in 8-cell stage embryos and selecting the appropriate transgenic animals, on which I performed double antibody staining against Pax3/7 (blue) and EGFP (green). tdTomato expression is in red. 7 out of 7 recorded satellite-like cells were mesodermal.

3.3 *Parhyale* satellite-like cells proliferate upon amputation and participate in the blastema

In vertebrates, upon amputation, satellite cells proliferate in order to replenish the satellite cell pool and to generate the cells that will eventually differentiate and fuse to form muscle fibers. I wanted to assess whether *Parhyale* SLCs responded to amputation by proliferating.

To identify whether these cells proliferate upon amputation, I amputated the appendages of adult animals and then exposed them to 0.2mM EdU in seawater for 6 hours, 12 or 24 hours post-amputation. Then I left the animals in normal seawater for another six hours before I performed the EdU staining. Figure 40A shows such staining 36 hours post-amputation. I could capture some muscle-associated cells that had started to proliferate. I also performed antibody staining for Pax3/7 in dissected appendages from animals, which had been treated with EdU as described above. In Figure 40B, a satellite-like cell, alongside with its progeny, can be seen. Unfortunately, these results were not conclusive due to limitations in the penetrance of the antibody posed by the exoskeleton. In the first case, the antibody did not penetrate; therefore, the presence of SLCs can only be judged by their position in the vicinity of muscle fibers. In the second case, I could only observe one Pax3/7-expressing cell.

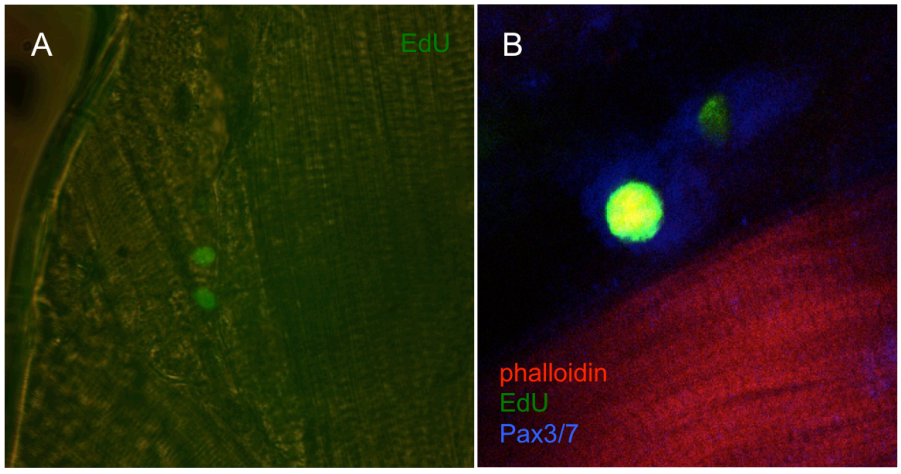


Figure 40: Satellite-like cells may proliferate following *Parhyale* leg amputation
(A, B) A pulse of EdU incorporation given as described earlier (labelled in green), reveals proliferating cells that are closely associated with muscle fibers, visualized by Nomarski (A) or phalloidin (B, red), and express Pax3/7 (B, blue). These cells may correspond to satellite-like cells described earlier. The antibody staining for Pax3/7 in adult appendages is hindered by the exoskeleton. Therefore, in the Pax3/7 and EdU double staining, I could identify only one Pax3/7-expressing cell.

To examine whether SLCs participate in the blastema, I exposed regenerating animals to EdU 2-3 days post-amputation for 6 hours and after a 6-hour recovery in artificial seawater, I stained the blastemas with the Pax3/7 antibody (Figure 41). The presence of three to four Pax3/7-expressing cells in the proximal part of the blastema, co-stained with EdU, suggests that satellite-like cells participate in the formation of the blastema and are actively proliferating. 2.5 Pax3/7-positive cells were scored in average in 12 blastemas. Satellite cells have also been found in the proximal part of the blastema in salamanders (Morrison, Loof et al. 2006).

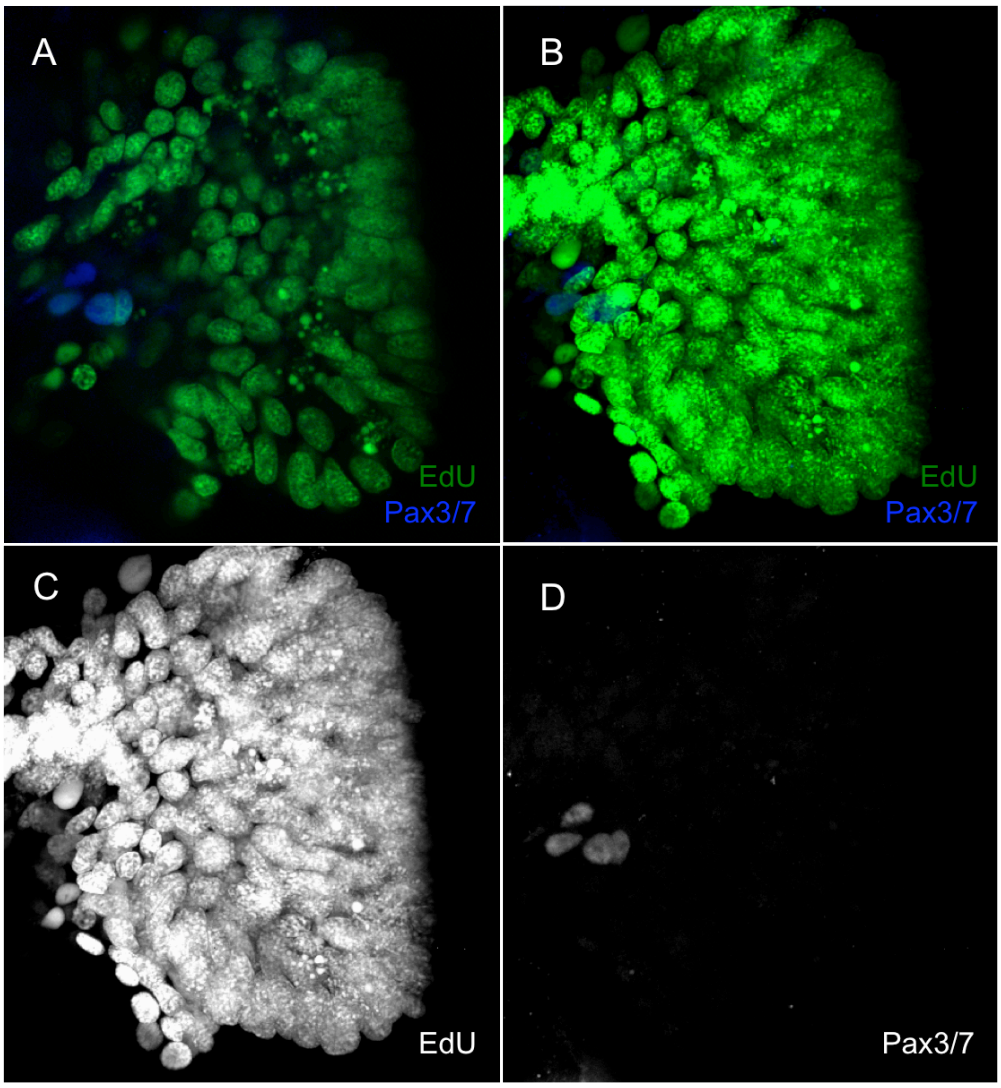


Figure 41: Satellite-like cells participate in the formation of the blastema
Satellite-like cells participate in the formation of the blastema, as indicated by the presence of 3-4 Pax3/7-expressing cells in 2-3 day post-amputation blastemas. An optical section and a maximum projection of a confocal scan are shown in (A) and (B), respectively. EdU staining was performed as described earlier (6 hours 0.2mM EdU pulse followed by 6 hour-recovery and, then, EdU staining). EdU is shown in green, Pax3/7 staining is shown in blue. In (C) and (D), individual channels of the maximum projection (EdU staining and Pax3/7 staining, respectively) are illustrated.

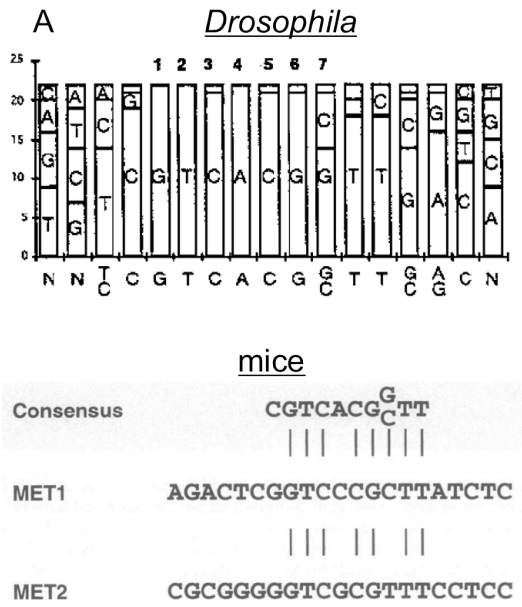
3.4 *Parhyale* satellite-like cells participate in appendage regeneration

Having established the presence of satellite-like cells and their activation upon amputation in *Parhyale* limbs, I wanted to examine their possible role as muscle progenitors during limb regeneration. I tried to answer this question using two experimental approaches.

Reporter construct

First, I tried to generate a Pax3/7 reporter construct. Having a promoter that responds to Pax3/7 driving the expression of a fluorescent protein would enable me to observe SLCs in living animals.. Moreover, I would be able to use this promoter to drive various genetic tools, for example the Flp/FRT constructs, in order to follow the descendants of SLCs and see whether SLCs act as progenitors during muscle regeneration. I could also combine this promoter with the cell-autonomous inducible killing system that was established in *Parhyale*, with the use of nitroreductase (Koltsaki 2012), to kill these cells and assess whether regeneration is compromised.

The recognition site of the paired domain of Pax3/7 is conserved in mice and *Drosophila* (Epstein, Shapiro et al. 1996; Jun and Desplan 1996). As shown in Figure 42A, there exists a consensus sequence, CGTCACGGTT, that responds to the presence of Pax proteins, and, especially, the paired domain.Since this sequence is conserved, I expected it should also be responsive in *Parhyale*. Therefore, I constructed a promoter sequence consisting of five tandem repeats of this sequence (Figure 42B), as previously described in *Drosophila* (Sheng, Harris et al. 1997; Sheng, Thouvenot et al. 1997) and combined this with several putative core promoter sequences. I cloned the binding site-core promoter construct upstream of a fluorescent protein in a Minos vector. If the construct was functional, Pax3/7, where it is expressed, would recognize its binding site and drive the expression of the fluorescent protein. I tried four different binding site-promoter combinations, but none was successful. I will briefly describe them.



B Parhyale construct

GGATCCGATCTTAGTC**GTCACGGTTCG**TTA
GGATCTTAGTC**GTCACGGTTCG**TTAGGATC
TTAGTC**GTCACGGTTCG**TTAGGATCTTAGT
C**GTCACGGTTCG**TTAGGATCTTAGTC**GTCA**
CGGTTCGTTAGGGATCCGGCGTGAAGGGA
GACTGAGATATAAGAAGCTGCTTCGCCGCC
AGCCTCTCAGTTTATCCTGAGCTTCTAATCG
CGATACATACTACCATTTTGCTCACACGAAA
GTAGAGAAAGGACTGGAATTTATTCTGTACC
CTGTGAATAATTTGCTGAAGTGTAGTTATTGT
CAAGAAAACCAATTTTATACTGTTTGATACC
TGTTCGAAAGACGCGAGTTCTGCGAAGTGA
TTTTTTAAGTCCTCTACATCACTTCAGCGCTA
CTGTGCCCGGGACTAGTAGATCTGTCGACCT
CGAGCCATGGGACCGCGGCCGCATCGTCTA
GAGGCTAAGCTT

Figure 42: Attempts to generate a Pax3/7 reporter
(A) In both flies and mice, the recognition site of the paired domain of members of the Pax3/7 family is highly conserved, displaying the core/consensus sequence CGTCACGG/CTT (images taken from (Epstein, Shapiro et al. 1996; Jun and Desplan 1996)). Constructs reporting the expression pattern of paired domain-containing transcription factors have been used in both species (Epstein, Shapiro et al. 1996; Sheng, Harris et al. 1997). (B) I constructed a potential Pax3/7-responsive element, consisting of five consecutive repeats of the conserved recognition site (sequence highlighted in green) and placed it upstream of the Drosophila *hsp70* basal promoter (putative TATA-box –TATAA- and transcription start site – A – highlighted in red). The translation start site is highlighted also in red – ATG – and useful restriction sites are highlighted in blue.

(a) I placed the Pax3/7 binding sites ~50 base pairs upstream of the *Drosophila* hsp70a basal promoter, which is also being used in the 3xP3 construct that we use in *Parhyale* as a transformation marker (Pavlopoulos and Averof 2005). This module was placed upstream of the DsRed coding sequence. Transgenic animals carrying this construct showed no expression of DsRed as embryos, hatchlings or adults.

(b) I placed the same construct including the Pax3/7 binding sites, *Dm*hsp70a basal promoter and DsRed in the same transposable element as the *Ph*HS element driving EGFP expression. We had noticed, in the past, that the presence of the *Ph*HS element in the vicinity of 3xP3 expanded the expression driven by 3xP3. This might be attributed to an influence of *Ph*HS on chromatin, surrounding the transgene. Transgenic animals carrying this construct showed no expression of DsRed as embryos, hatchlings or adults.

(c) Supposing that the inefficiency of the constructs is due to the exogenous basal promoter, I decided to replace the *Dm*hsp70a basal promoter with a *Parhyale* basal

promoter. I had noticed during my Master thesis that in the presence of *Ph*HS basal promoter (i.e. the *Ph*HS regulatory element without the Heat-Shock Factor binding sites) the expression of 3xP3 was expanded from a single spot laterally of the eyes to the whole central nervous system. Therefore, I used the *Ph*HS basal promoter in combination with the Pax3/7 binding sites. Transgenic animals carrying this construct showed no expression of DsRed as embryos, hatchlings or adults.

(d) I decided to use a basal promoter that was already known to be functional in the mesoderm; this was the basal promoter of the *Ph*MS element. I, therefore, replaced the array of putative MyoD binding elements that are upstream of the transcription start site (Kontarakis, Pavlopoulos et al. 2011) with the paired domain binding elements. In this case, I recorded expression in the whole musculature. As a control, I used the *Ph*MS element without the upstream putative MyoD binding sites. Surprisingly, I observed that this fragment was also capable of driving expression in the whole musculature and, hence, the upstream putative MyoD binding sites seem not to be necessary for the promoter's activity.

After these efforts, I decided to abandon the quest for a reporter construct.

Transplantation experiments

The second approach I took was the transplantation of marked satellite-like cells into a regenerating appendage. If, for example, the satellite-like cells from an animal carrying the *Ph*MS-EGFP transgene could be transplanted into a wild type animal, I would be able to track these cells and their descendants by following the expression of EGFP and test whether satellite-like cells can directly contribute to the regeneration of muscle fibers. If I was not able to detect any fluorescence, I could not be sure whether this was a consequence of the unsuccessful transplantation or of the fact that satellite-like cells do not participate in appendage muscle regeneration.

The first step was to isolate these cells. I used animals carrying the *Ph*MS-EGFP transgene (Pavlopoulos and Averof 2005). I could tell these cells apart because the muscle fibers have a very distinct elongated shape, whereas the satellite-like cells are compact circular cells. I tried dissecting limb muscles out and homogenizing them in order to separate cells and pick the ones that are fluorescent and circular, but I could isolate only a few satellite-like cells. Eventually, I worked out an efficient way of

isolating satellite-like cells, which works as follows: appendages were cut into pieces, treated with 0.25 mg/ml of collagenase in Shields and Sang M3 medium for an hour in room temperature. For all the experiments I treated cells with collagenase in room temperature, instead of 37^{o}C where collagenase is more active, to avoid any temperature challenge of the cells. Then, carefully, I dissected these cells out of the appendage. The muscle-fiber bundles are attached to the cuticle, so if the cuticle is smoothly pressed and pushed towards one end, the satellite-like cells can be extracted successfully, whereas the rest of the fluorescent tissue stays within the cuticle. Then, these cells can be picked up with a microinjection needle and placed in a separate vial.

To verify that these cells are indeed satellite-like cells, I performed antibody staining against Pax3/7 on cells isolated as described earlier from animals carrying the *Ph*MS-EGFP transgene. The isolated cells were spun on a microscope slide using a Cytospin 4 centrifuge (Thermo Scientific). After fixation, I combined antibody staining against Pax3/7 and EGFP with DAPI staining. I verified that, among the isolated cells, all the cells that express EGFP, express also Pax3/7, whereas EGFP-negative cells do not. Figure 43A shows two cells, one expressing EGFP and Pax3/7 and one not. I quantified the expression levels of Pax3/7 in EGFP-positive and EGFP-negative cells, using Fiji. I also performed a control experiment, where I omitted the Pax3/7 antibody during the staining. Figures 43B and 43C show two independent quantification experiments. In each experiment, the data points, which represent fluorescent intensity in one cell, were quantified from images obtained the same time and under the same settings. Therefore, I concluded that the cell isolate consists of EGFP-expressing Pax3/7-positive satellite-like cells and EGFP-negative, Pax3/7-negative cells.

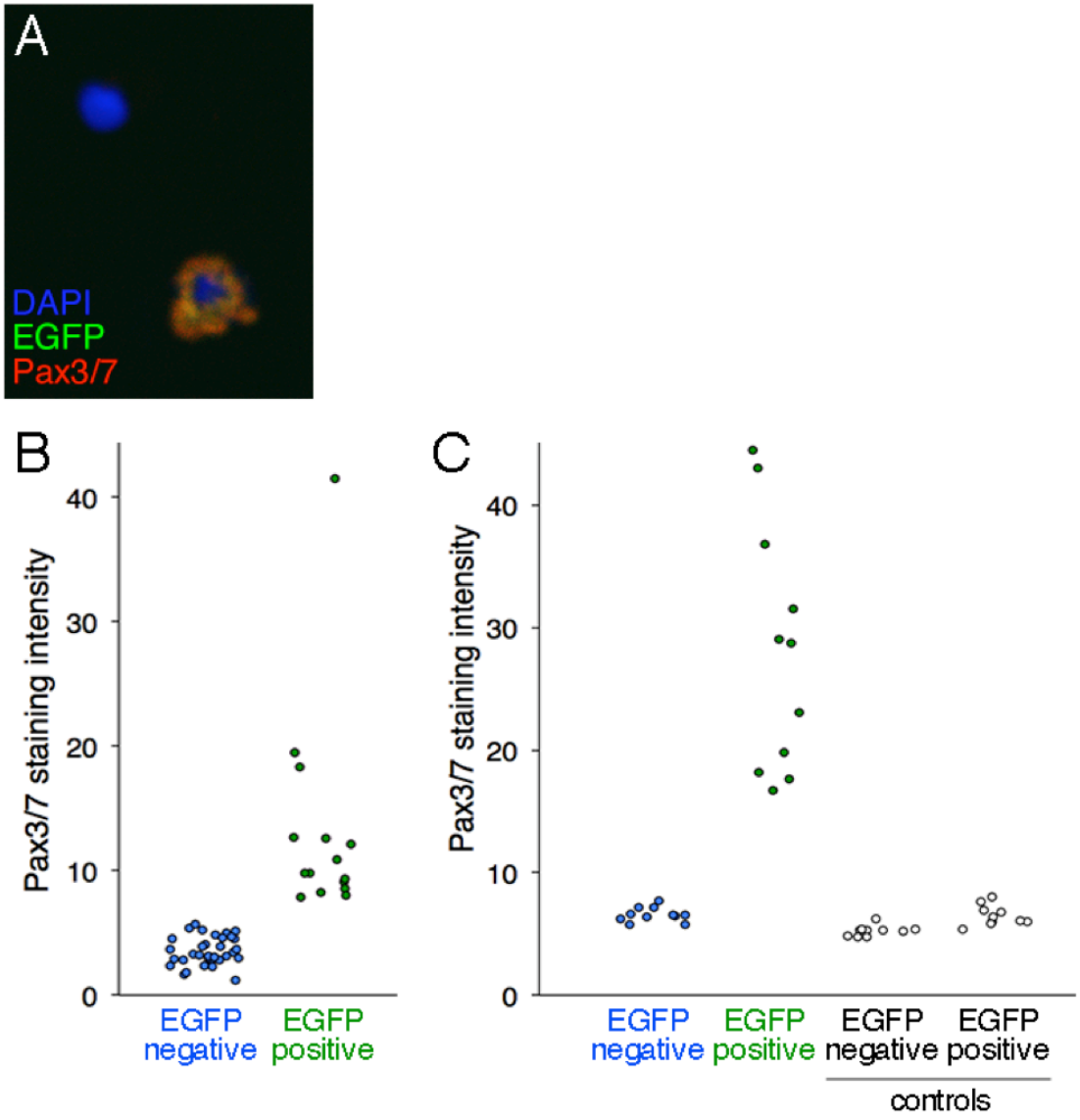


Figure 43: The isolated EGFP-positive cells are satellite-like cells
To prove that the EGFP-positive cells I isolate are indeed satellite-like cells, I spun the isolate in a Cytospin 4 centrifuge to make them adhere on a microscope slide. After fixation, I combined double antibody staining for EGFP and Pax3/7 with DAPI staining. (A) The EGFP-expressing cell (green) is also expressing Pax3/7 (red), contrary to the non-EGFP-expressing cell (stained only with DAPI; blue). (B, C) The Pax3/7 expression levels from EGFP-positive and EGFP-negative cells were quantified using Fiji. Two independent experiments are displayed. In the second one (C), I included a negative control where I omitted the primary antibody against Pax3/7 during the staining. EGFP-expressing cells are all expressing Pax3/7 in variable levels, but always above background. EGFP-negative cells display background levels of Pax3/7 staining. Each point represents the Pax3/7 expression levels in one cell, measured in arbitrary units.

After achieving the isolation of satellite-like cells, I attempted to transplant these cells in appendages, either before or after amputation. I transplanted 5-15 satellite-like cells per appendage. I tried transplanting these cells in different appendages. The easiest way to do it was to transplant the cells in a freshly amputated thoracic appendage,

amputating the carpus of the fourth or fifth thoracic appendage and transplanting the cells immediately after amputation. Out of 105 animals that received satellite-like cells in one appendage, 72 survived until regeneration. I tested these animals for EGFP-expressing muscle fibers 45 days after amputation. In 12 of them, I could record the presence of EGFP-expressing muscle fibers upon regeneration in the amputated appendage (Figure 44). In all cases, the EGFP-expressing fibers were in the carpus of the appendage (in 6 cases in the fourth, and in 6 cases in the fifth thoracic appendage), i.e. the podomere where I had transplanted them. Only one animal had more than one EGFP-expressing muscle fibers. Most of the times (11 out of 12 animals), a single fiber was expressing EGFP.

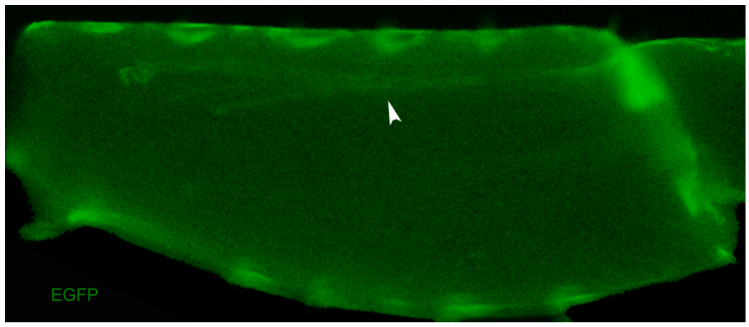


Figure 44: EGFP-expressing muscle fibers in the recipient appendage derived from transplanted SLCs
After transplantation of satellite-like cells from transgenic animals carrying the PhMS-EGFP reporter into the amputated leg of a wild-type recipient, and subsequent regeneration, I observed weak green fluorescence within a few muscle fibers (arrowhead), above the background of green autofluorescence from the leg's exoskeleton. Subsequent staining of this limb with an antibody for EGFP (figure 45A) confirmed that these are EGFP-expressing muscle fibres

To test whether these fibers could derive from the EGFP-negative cells that were in the isolate and were transferred in the amputated appendage alongside with the satellite-like cells, I performed a control experiment where only EGFP-negative cells were transplanted from the MS-EGFP transgenic animals in wild-type recipients. After dissecting satellite-like cells out of cut appendages, as described earlier, I picked cells, exactly as I did in the actual experiment, with the only difference that I omitted picking EGFP-expressing cells. I proceeded with the transplantation of this isolate into wild-type appendages, exactly as described before. Out of 50 animals that received EGFP-negative cells in one appendage, 34 survived until regeneration and were inspected for EGFP-positive muscle fibers 45 days after amputation. In none of

these cases could I recognize EGFP-expressing muscle fibers in the regenerated limbs.

To confirm that the green fluorescence of the fibers is due to EGFP expression (and are, therefore, progeny of the transplanted satellite-like cells), I performed antibody staining against EGFP. I combined the antibody staining with phalloidin staining to verify that these fibers are indeed muscle fibers and with DAPI to see if they are multinucleated, as is the case for wild-type muscles (Figure 45). Figure 45A shows that the muscle fiber that was also shown in Figure 44 is indeed expressing EGFP and is bound by phalloidin. The same is true for the muscle fiber, which is transversely sectioned and stained with EGFP, phalloidin and DAPI, in Figure 45C. In Figure 45B, a close-up of the transgenic muscle fiber shows that this fiber is multinucleated. The previous experiments show unambiguously that satellite-like cells can operate as progenitor cells for regenerating muscle.

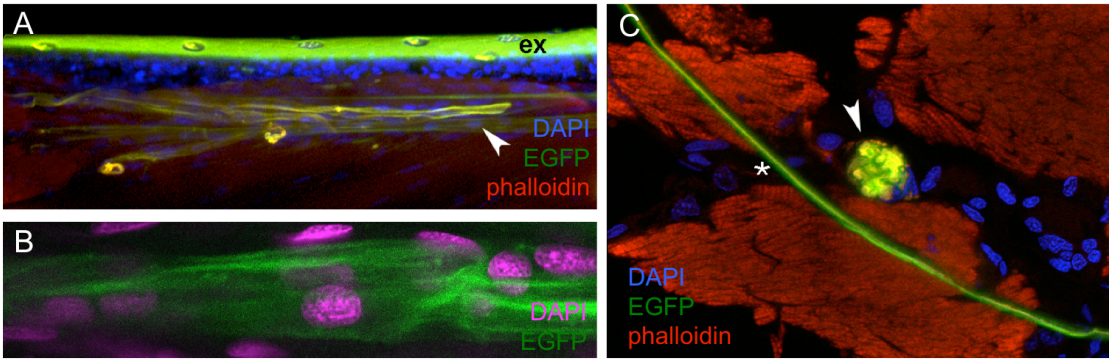


Figure 45: Transplanted satellite-like cells contribute to muscle fibers in regenerated thoracic legs
12 out of 72 appendages, that had been amputated, transplanted with SLCs and left to regenerate, carried EGFP-expressing muscle fibers. (A) The satellite-like cell-derived muscle fibers (arrowhead) can be detected by EGFP fluorescence (green), in appendages stained also with phalloidin (red) and DAPI (blue). ex indicates autofluorescence of the cuticle. (B) Close-up of the same muscle fiber shows that it is multinucleated. (C) Transverse sections of these appendages show that the satellite-like cell-derived muscle fibers (arrowhead) express EGFP (based on antibody staining against EGFP; green) and stain with phalloidin (red). Asterisk indicates autofluorescence of a piece of cuticle that was misplaced during sectioning.

Omnis cellula e cellula (Where there is a cell, there has been a cell).

Rudolph Virchow (1855)

Chapter 4

Conclusion and perspectives

Regenerative capacity varies among cells, tissues, organs and species. Therefore, it is difficult to approach it as a whole. In this thesis, I focused on regeneration as the restoration of a tissue that consists of cells of different lineages. In this context, data from this and previous studies indicate that in the Metazoans most of the regenerating processes are governed by progenitor cells that have a restricted regenerative potential, a remarkable exception being the flatworms. The muscle progenitors in particular, the muscle satellite cells seem to be involved in restoring damaged muscle, both in vertebrates and arthropods. In this Chapter, I will summarize the main conclusions that can be drawn from my work and try to present, what I believe are interesting goals to be achieved in the future.

4.1 Conclusion: Common cellular mechanisms during limb regeneration in arthropods and vertebrates

My work has highlighted two main similarities between vertebrate and arthropod limb regeneration: the participation of pre-determined progenitors in appendage regeneration and the participation of satellite-like cells in muscle regeneration.

Lineally restricted and regionally active progenitors

Parhyale achieves regeneration of its limb tissues by employing two different pools of progenitors, one to regenerate the ectodermal and one to regenerate the mesodermal tissues. In addition, these progenitors appear to reside locally, close to the regenerating tissue. This type of regeneration progenitors resembles significantly the type of progenitors that participate in vertebrate limb regeneration (Gargioli and Slack 2004; Kragl, Knapp et al. 2009; Rinkevich, Lindau et al. 2011; Tu and Johnson 2011) and differs from the totipotent progenitors that drive regeneration in planarians (Reddien and Sanchez Alvarado 2004; Wagner, Wang et al. 2011).

The use of fine clonal analysis tools, such as Brainbow (Livet, Weissman et al. 2007), would help discover the progenitor cells within each pool that are important for regeneration of specific tissues. As will be discussed next, satellite-like cells participate in *Parhyale* muscle regeneration, but it would be interesting to know which progenitor cells are responsible for the regeneration of the epidermal cells, the nerve cells and other cell types.

Satellite-like cells

The discovery of muscle satellite cells that express Pax3/7 in *Parhyale* marks the first time such cells are discovered outside the chordate lineage. Only recently, satellite cells were identified outside vertebrates, and, specifically, in *Amphioxus* (Somorjai, Somorjai et al. 2012). In the past, the presence of satellite cells in decapod crustaceans' cardiac muscles had been proposed, based on morphological similarities to their vertebrate counterparts (Midsukami 1981), but my study is the first to rigorously demonstrate the presence of satellite cells expressing the molecular marker Pax3/7 and participating in appendage regeneration.

The discovery of satellite-like cells in *Parhyale* pushes back the origin of satellite cells to the last common ancestor of chordates and arthropods. Moreover, their participation in muscle regeneration implies that the ability to regenerate muscles dates back to precambrian times. Alternatively, muscle regeneration may have evolved independently in arthropods and vertebrates, engaging a homologous cell type – satellite cells – as the source of regenerated muscles. In the latter case, the ancestral satellite cells may have been involved in adult muscle growth, homeostasis and repair, which would have pre-adapted them for a role in regeneration.

Several questions regarding the exact role of satellite-like cells during *Parhyale* muscle regeneration in comparison to their role in vertebrates should now be addressed. Are *Parhyale* satellite-like cells the only contributors to muscle regeneration, or do dedifferentiated muscle fibers participate as well? Are they necessary and/or sufficient? How heterogeneous is the satellite-like cell population? Which is the molecular fingerprint of satellite-like cell activation? Utilization of genome engineering techniques, such as zinc-finger nucleases (Porteus and Carroll 2005), TALENs (Cermak, Doyle et al. 2011) and CRISPR/Cas9(Cong, Ran et al. 2013), to gain access to the genomic locus of *Ph*Pax3/7-2 and mark satellite-like cells in combination with clonal analysis tools, inducible cell-killing tools and new-generation transcriptome sequencing techniques could help to assess these questions.

4.2 Perspectives: Mid-term goals

What triggers regeneration?

It has been proposed that upon injury in vertebrates, there are two ways of dealing with the wound: either the formation of a fibrotic scar through an inflammatory response or tissue regeneration (Mescher and Neff 2005; Godwin, Pinto et al. 2013). Within a few days upon injury in mice, macrophages accumulate at the wound site and can acquire two different phenotypes, an inflammatory and an anti-inflammatory, which can be distinguished from each other based on gene expression (Ruffell, Mourkioti et al. 2009). Forcing macrophages to acquire an inflammatory phenotype during mouse muscle repair leads to defective musculature and fibrotic scarring (Ruffell, Mourkioti et al. 2009). Moreover, it has been observed that macrophage ablation during salamander limb regeneration permits wound closure but prevents

regeneration (Godwin, Pinto et al. 2013). It seems that, in vertebrates, the immune response could play a role as a regeneration trigger

The role of apoptosis as an early regeneration signal has been discussed extensively in the past (Bergmann and Steller 2010, Mescher, White and Brokaw 2000, Pellettieri… and Alvarado 2009) without a rigorous answer as to its role in promoting or inhibiting regeneration. During *Xenopus* tail regeneration, apoptosis is necessary in order for regeneration to proceed; when apoptosis is inhibited, regeneration is eliminated (Tseng, Adams et al. 2007). Similar results have been observed in mice, flies and *Hydra* (Bergmann and Steller 2010) supporting the notion that apoptosis is not regeneration-specific, but, more generally, implicated in repair. Contrary to this idea, studies in chick, which is capable of regenerating its spinal cord until embryonic day E13, associate the loss of regenerative capacity with the expression of a cell death mediator and with increase of apoptosis (Lange, Gogel et al. 2011).

I believe it would be very interesting to study different animals that are capable of regenerating and discover what triggers regeneration in each case. We would, thus, understand whether different species share common regeneration trigger mechanisms and whether these mechanisms can potentially explain the differences in the regenerative capacity between animals.

Regeneration at molecular resolution

The molecular basis of regeneration is largely unexplored. Most of the information that are available for gene participation during regeneration are based on a candidate gene approach. From such studies several conserved signaling pathways have been identified to participate during regeneration in different systems. For example, Wnt signaling has been proven to be very important during regeneration in planarians, *Hydra*, *Tribolium,* zebrafish, axolotl and *Xenopus*(Kawakami, Rodriguez Esteban et al. 2006; Stoick-Cooper, Weidinger et al. 2007; Gurley, Rink et al. 2008; Galliot and Chera 2010; Shah, Namigai et al. 2011). The same stands for BMP, FGF and other pathways. Since all of these pathways are conserved and participate actively during development for proliferation and patterning, it is a priori highly likely that they will also be involved in regeneration. The importance of knowing the contribution of these signaling cascades in regeneration should not be underestimated, however the candidate gene approach will never reveal regeneration-specific mechanisms.

New-age sequencing technologies can offer an unbiased approach for discovering regeneration-specific genes. RNA sequencing at different time-points, from different tissue samples and species can provide an insight as to the sets of genes that are expressed during regeneration and/or scar formation. Efforts have been made in this direction especially in planarians (Blythe, Kao et al. 2010; Sandmann, Vogg et al. 2011), but the field is still in its infancy. Moreover, with the recent developments in sequencing technology, it is now possible to identify key genes that might be expressed at lower levels and study qualitative differences in gene expression in single-cell resolution.

Combinations of genetic techniques and high-throughput technologies are bound to make molecular studies of regeneration a very attractive field of study in the following years. *Parhyale* as a genetic and regeneration model can serve as a crustacean representative in such studies. Apart from the genetic techniques and the sequencing approaches that can be employed in *Parhyale*, the gene-trapping technique and iTraC (described in Chapter 1) can offer an unbiased approach for identifying genes that participate in limb regeneration and studying them in detail.

4.3 Perspectives: Ultimate goals

Understanding the evolutionary history of regenerative capacity

The irregular distribution of regenerative capacity in the tree of life poses questions concerning the evolution of regeneration. It is fascinating that there exist very closely related species, such as *Lineus ruber* and *Lineus viridis*, (mentioned earlier) that differ strikingly in their regenerative capacities. Among 24 urodele species studied for their regenerative capacity, a few had no regenerative capacity whereas the others were capable of regenerating legs and tail (Brockes, Kumar et al. 2001). Similarly, flatworms with different regenerative capacities have been identified and studied (Reddien and Sanchez Alvarado 2004). Consequently, the question that arises is whether regeneration is an ancient trait that was lost in some lineages or whether it has evolved independently multiple times.

If regeneration were an ancient trait, it would inevitably generate the question of why it has been lost in so many species, since intuitively it seems advantageous for every organism.

One reason could be that loss of regenerative capacity might not be directly under selection; instead, it might be an epiphenomenon of some other trait, which in certain cases has been selected against. For example, the existence of cells that can, upon activation, proliferate might make the organism more likely to develop cancer. In fact, in newts, carcinogen administration may cause, at the same time and in the same eye, lens regeneration from the dorsal iris and tumor production from the ventral iris (Okamoto 1997).

Alternatively, although regeneration might be adaptive, it might be incompatible with another adaptive trait; hence, in every organism only one of the two traits can be present. Rapid scar formation may be adaptive. It seems that the formation of a scar is incompatible with early events of regeneration. So, in some organisms it might be more important to be able to form a scar, which can heal the wound more rapidly, than investing time and energy in regenerating a new appendage. This has been proposed to be the reason why warm-blooded vertebrates lost their capacity to regenerate (Brockes, Kumar et al. 2001).

Another possibility is that some animals might have employed different strategies to maximize their reproductive capacity that render regeneration neutral for evolution. If for these animals regenerative capability is not increasing reproductive capacity, it will be lost.

On the other hand, independent evolution of the regenerative capacity in different species might be easier than thought. The mechanisms of producing a new limb are already encoded in the animals' genomes, as they have been used during embryonic development. Therefore, in order for an animal to evolve the capacity to regenerate a limb, it might just need to find a way to trigger the re-utilization of these mechanisms.

Studying natural phenomena, such as the regeneration in frogs, which are able to regenerate as tadpoles but not as adults, or regeneration of newt lens, which can regenerate lens cells from dorsal but not from ventral iris pigment epithelial cells or closely related species that differ significantly in their regenerative capacities, such as *Lineus riber* and *Lineus viridis*, and accumulating information from diverse species that are able to regenerate can help us approach this question more systematically.

Comprehension of cell plasticity

A major goal in stem cell therapy nowadays is to to derive all kinds of cells from committed precursors. Differentiation is a multifactorial process that is hard to be reversed. By transdifferentiating an already committed cell into another, one needs to make sure that the produced cell has no memory of its previous state of commitment. The ideal scenario, in my mind, would be to identify natural phenomena of dedifferentiation and, especially, transdifferentiation and study how nature achieved this goal.

Regeneration, obviously, offers a framework where one could identify such cases. Transdifferentiation has been observed during tail regeneration in salamanders (Echeverri and Tanaka 2002), in echinoderm gut regeneration (Mashanov, Dolmatov et al. 2005), in regeneration of pancreatic beta-cells (Thorel, Nepote et al. 2010) and newt lens regeneration (Del Rio-Tsonis and Tsonis 2003) but, generally, it does not seem to be a common mechanism. Moreover, the dedifferentiation phenomena that have been observed, for example during muscle or bone regeneration (Jopling, Sleep et al. 2010; Knopf, Hammond et al. 2011), are not dramatic; the cells dedifferentiate to a committed state where they proliferate to generate tissue of the exact same lineage. However, since the tools for rigorous proof of dedifferentiation and/or transdifferentiation phenomena, such as Cre/lox and Flp/FRT, have recently been employed in regenerating species, it is likely that they can offer a chance of identifying more such cases that will provide much needed material for further stem cell research.

To reach these goals, it is necessary to answer more fundamental questions about regeneration; to expose the evolutionary history of regeneration, identify the common and different elements between regeneration and embryonic development, comprehend the complexity of this process and acknowledge the similarities and differences between regenerative processes in diverse contexts (cells, tissues, organs etc). This thesis has introduced a new regeneration model and described basic aspects of regeneration in this model, hopefully moving the regeneration field one step closer to its ultimate goals.

"And the whole strenuous intellectual work of an industrious research worker would appear, after all, in vain and hopeless, if he were not occasionally through some striking facts to find that he had, at the end of all his criss-cross journeys, at last accomplished at least one step which was conclusively nearer the truth."

Max Planck, 1920, Nobel Lecture

Appendix A

Materials and methods

A.1 Molecular Biology Techniques

1.1 Cloning in plasmid vectors

a) DNA and RNA handling

Agarose gel electrophoresis was applied for the separation, identification and extraction of DNA fragments. Ethidium bromide was used for the staining of DNA and the extraction and purification of DNA fragments was performed with commercial kits (Qiagen, Nucleobond). The final concentration of nucleic acid solutions was determined with the Nanodrop. The nucleic acid restriction and modification enzymes were from the companies Minotech, New England Biolabs, Ambion and Promega. Reactions were performed in the conditions recommended by the manufacturer. DNA fragment sequencing was performed by the Microchemistry lab of IMBB or by Macrogen.

b) Preparation of competent *E. coli* cells

1. Cells were streaked out on LB plates without antibiotics. The plates were incubated at 37°C over night.
2. One colony was picked and used to inoculate 5 mL LB, which was incubated for approximately 8 h.
3. 200 mL selective LB was inoculated with 2 mL of the starter culture and incubated at 37 °C by vigorous shaking until an optical density (OD_{550}) of 0.4-0.6 was reached.
4. The culture was split in 4 parts and each part was spun down at 2500 rpm at 4 °C for 20 min.
5. Each pellet was resuspended in 15 mL Tpb I buffer and incubated on ice for 30 min.

6. The cells were pelleted at 2500 at 4 °C for 10 min.
7. The pellets were resuspended altogether in 8 mL Tpb II buffer.
8. The cells were split into 100 µL aliquots into pre-cooled 1.5 mL reaction tubes and frozen in liquid nitrogen. The cells were stored at -80 °C.

c) Transformation of chemically competent cells

1. An aliquot of frozen cells (approximately 100 µL) was thawed on ice.
2. An appropriate amount of DNA, depending on the specific reaction setup, was added to the cells.
3. The tubes were placed on ice for 30 min.
4. Heat shock was performed at 42 °C for 90 s in a water bath, after which the cells were quick-chilled on ice.
5. 500 µL LB were added and the tubes incubated shaking at 250 rpm at 37°C for 1 h.
6. The bacteria were spread on selective plates and incubated at 37°C over night.

d) Plasmid mini-preparation by alkaline lysis

1. 2 mL of an overnight *E. coli* culture were spun down at 13000 rpm for 2 minutes.
2. The pellet was resuspended in 100 µL Solution I with 20 µg RNase.
3. 200 µL Solution II were added and the samples were incubated on ice for 5 min.
4. 150 µL Solution III was added and incubated on ice for 10 min.
5. The sample was centrifuged at 13000 rpm for 15 minutes at 4^{o}C.
6. The supernatant was mixed with an equal amount of phenol, vortexed and spun down at 13000 rpm for 5 min.
7. The supernatant was mixed with an equal amount of chloroform, vortexed and spun down at 13000 rpm for 5 min.
8. Then, the DNA was precipitated by adding 1/10 volumes sodium acetate (3 M, pH=5.2) and 2 volumes of prechilled ethanol to the supernatant followed by centrifugation at 13000 rpm for 15 min.
9. The pellet was washed with 70 % ethanol and centrifuged for 5 min.
10. The pellet was air-dried and resuspended in 20 µL water. The DNA concentration was measured with the NanoDrop®.

e) Plasmid midi-preparation

The midi-preparation was performed with the Macherey-Nagel Nucleobond Xtra Midi kit, according to the manufacturer's protocol.

1.2 Nucleic acid extraction from *Parhyale*

a) Genomic DNA extraction

1. Collect one adult or ten embryos and freeze them.
2. Add 150 ul of grind buffer (0.1M Tris pH 9.2, 0.2M Sucrose, 50mM EDTA, 0.5% SDS) to the animals and homogenize.
3. Rinse debris off the pestle into tube with 150 ul more of grind buffer
4. Vortex tube hard for 10 sec. and place immediately in 65c water bath for 30 min.
5. Add 600 ul 5M KAc and vortex briefly to mix, place on ice at least 15 min.
6. Add phenol/chloroform, vortex and spin for 15 min at 4^{o}C. before centrifugation in order to have cleaner product.
7. Transfer the supernatant by pipetting into an eppendorf containing 400 ul isopropanol. Mix well.
8. Centrifuge the tube 15 min at 13000 rpm.
9. Wash pellet with 70% EtOH, spin, remove all liquid from pellet, air dry.
10. Add 80 ul H2O to pellet.

b) Total nucleic acid extraction (Holmes-Bonner)

1. Collect embryos in a tube, remove seawater as much as possible.
2. Add 100ul HB buffer (100mM Tris pH7.5, 10mM EDTA, 300mM NaCl, 2% SDS, 7M Urea) and homogenize for 1 minute.
3. Add 100ul HB buffer and 200ul phenol/chloroform and mix carefully for 10 minutes.
4. Spin at 13000 rpm for 5 minutes at room temperature.
5. Transfer supernatant in a new eppendorf.
6. Phenol/chloroform extraction. Repeat three times.
7. Chloroform extraction. Repeat twice.
8. Ethanol precipitation.
9. Wash pellet with 70% EtOH, spin, remove all liquid from pellet, air dry.
10. Resuspend in 20ul ddH_2O.

11. Perform PCR using 5ul of the extracted plasmid.

c) Total RNA extraction

1. Collect embryos in a tube, remove seawater as much as possible.
2. Add 150ul Trizol and grind well for a minute.
3. Add 150ul Trizol and grind for another minute.
4. Vortex for 1 minute.
5. Add 60ul chloroform and spin at top speed 5 min.
6. Transfer supernatant into a new eppendorf and add 150ul isopropanol.
7. Spin at top speed for 40 min
8. Wash pellet with 70% EtOH, spin, remove all liquid from pellet, air dry.
9. Resuspend in 20ul RNase-free ddH_2O.

A.2 Stainings

2.1 Antibody stainings

a) Antibody staining in *Parhyale* embryos

1. Dissect embryos in 4% Formaldehyde in Sea Water (complete dissection in 15 minutes).
2. Rinse embryos twice in PTx drops (1xPBS, 0.1% TritonX).
3. Transfer them in 2ml tubes and wash them for 5 minutes in PTx.
4. Block at 4°C for 1 hour in PTx with 5% Normal Goat Serum (NGS).
5. Add antibody and leave at 4°C overnight.
6. Rinse twice in PTx.
7. Wash in PTx for 30 minutes. Repeat four times.
8. Block at 4°C for 1 hour in PTx with 5% Normal Goat Serum (NGS).
9. Add secondary antibody and leave at 4°C overnight. Add phalloidin, TOPRO or DAPI if needed.
10. Rinse twice in PTx.
11. Wash in PTx for 30 minutes. Repeat four times.

i) Fluorescence-coupled secondary

12. Add 70% glycerol and mount on microscope slide

ii) AP-coupled secondary

12. Wash three times for 5 minutes with freshly made AP buffer (100 mM Tris-Cl pH 9.5, 100 mM NaCl)
13. Prepare fresh BCIP/NBT reaction solution (1ml AP reaction buffer pH 9.5, 5µl NBT 100mg/ml in 70% DMF, 3.75µl BCIP 50mg/ml in 100% DMF)
14. Add BCIP/NBT reaction solution
15. Develop in dark (check after 15-30', the reaction can be refreshed by replacing reaction solution after 3-4 hours)
16. Rinse twice with PTx
17. Wash twice for 10 minutes with PTx

ii) HRP-coupled secondary

12. Replace PTx with 300ul DAB solution (0.3ug/ul DAB in PTw)
13. Add 15ul 1% NiCl, incubate for 5 minutes
14. Add 15ul 0.3% H2O2
15. Develop for 10-15 minutes
16. Rinse twice PTx
17. Wash PTx overnight

b) Antibody stainings in adult *Parhyale*

Because of the exoskeleton, in order to perform antibody stainings in adult animals or appendages of adult animals, I performed cryosections prior to the staining. The animals or the appendages were fixed for 30 minutes in 4% formaldehyde, rinsed with PTx twice, washed with PTx twice for 10 minutes and then incubated in 15% sucrose solution in PBS overnight prior to the sections. Subsequently, they were embedded in gelatin-sucrose solution (1x PBS, 7,5% w/v gelatin (Sigma, G-2500), 15% w/v sucrose). The embedded animals/appendages were frozen in an ethanol bath and then were sectioned in a cryotome. The cryosections were 10-14um thick.

The staining protocol is as follows:

1. Hydrate the sections with PTx for 5 minutes.
2. Remove PTx carefully and add 150ul of blocking buffer (1x PTx, 5% NGS), incubate for 1 hour.
3. Remove blocking buffer and and add the first antibody solution.

4. Incubate at 4°C overnight.
5. Wash sections with PTx for 5 minutes. Repeat three times.
6. Add the secondary antibody solution.
7. Incubate for 2 hours at room temperature.
8. Wash sections with PTx for 5 minutes. Repeat twice.
9. Add TOPRO or DAPI (if needed). Incubate for 5 minutes.
10. Wash sections with PTx for 5 minutes. Repeat twice.
11. Add 70% glycerol and place the cover slip.

2.2 EdU staining

EdU staining was performed using the Click-iT AlexaFluor488 kit of Invitrogen.

1. Animals are placed in 0.2mM -containing artificial seawater for 6 hours.
2. Tranfer animals in seawater for 6 hours prior to staining.
3. Amputate appendages and fix them in 4% formaldehyde in artificial seawater for 20 minutes at room temperature.
4. Rinse the appendages twice in 500ul of PTx at room temperature.
5. Wash again in PTx twice for 20 minutes at room temperature.
6. Add 500ul of blocking solution (1x PTx, 5% NGS) and incubate for 30 minutes at room temperature.
7. Wash in PTx at room temperature.
8. Prepare the Click-iT reaction mix as instructed by the manufacturer.
9. Add 100ul of Click-iT reaction mix and incubate for 30 minutes at room temperature.
10. Wash appendages twice in PTx at room temperature.
11. If needed, proceed for antibody staining as described in (a).
12. Add 70% glycerol.

2.3 *In situ* hybridization

DAY 1

1. Collect embryos of appropriate stages and rinse them four times with ddH_2O.
2. Add prechilled 4% formaldehyde in ddH_2O and fix overnight at 4°C.

DAY 2

3. Rinse embryos three times with PBS

4. Wash with PBS for 6hrs

5. Dissect embryos (alternatively replace PBS and leave O/N)

6. Rinse twice with PTw (1xPBS, 0.1% Tween-20)

7. Fix with 4% formaldehyde in PTw for 30'

8. Rinse twice with PTw

9. Wash twice for 10 minutes with PTw

10. Wash for 30' with detergent solution (50mM Tris-HCl pH 7.5, 1mM EDTA pH 8.0, 150 mM NaCl, 1% SDS, 0.5% Tween-20)

11. Rinse twice with PTw

12. Wash four times for 10 minutes with PTw

13. Transfer embryos into baskets

14. Wash with 50% Hyb buffer (50% formamide, 5x SSC, 50ug/ml heparin, 0.25% Tween-20, 1% SDS, 100ug/ml salmon sperm DNA) in PTw for 10 minutes (20x SSC: 3M NaCl, 0.3M Sodium citrate, pH to 7.0 and sterilize by autoclaving, pH to 4.5 prior to use with HCl)

15. Wash with Hyb buffer for 10 minutes

16. Incubate in fresh Hyb for more than 3 hours at 65° C

17. Incubate stock probe solution (100ng/ul in Hyb) at 42°C for 10 minutes, mix and spin down

18. Dilute each probe to a final concentration of 1ng/ul in 1.5ml Hyb buffer

19. Denature probes at 80-90°C for 20 minutes

20. Transfer embryos into well with probe

21. Incubate 65° C 30-40hrs

DAY 4

22. Preheat Hyb buffer at 65° C and aliquot into wells

23. Wash three times for 20 minutes in Hyb buffer at 65° C

24. Wash four times for 30 minutes in Hyb buffer at 65° C

25. Wash twice for 5 minutes in preheated Hyb buffer at room temperature

26. Wash three times in TBST (125 ml 1M Tris pH 7.5, 40 g NaCl, 1g KCl, 5 ml 10% Tween-20, add ddH_2O until 500 ml - remove to well containing 0.75ml TBST and add 0.75ml of previous solution) for 30 minutes

27. Wash three times for 20 minutes in TBST
28. Block with TBST with 0.1% BSA at 4° C for 1hr
29. Transfer into well with primary antibody
30. Incubate at 4°C overnight

DAY 5

31. Wash four times for 30 minutes with TBST
32. Wash for 1 hour with TBST
33. Wash three times for 5 minutes with freshly made AP buffer (100 mM Tris-Cl pH 9.5, 100 mM NaCl)
34. Prepare fresh BCIP/NBT reaction solution (1ml AP reaction buffer pH 9.5, 5µl NBT 100mg/ml in 70% DMF, 3.75µl BCIP 50mg/ml in 100% DMF)
35. Transfer into well with BCIP/NBT reaction solution
36. Develop in dark (check after 15-30', the reaction can be refreshed by replacing reaction solution after 3-4 hours)
37. Rinse twice with TBST
38. Wash twice for 10 minutes with TBST

A.3 Isolation and handling of *Parhyale* embryos

Pregnant females were identified carrying the embryos in the brood pouch. They were anaesthetized by transferring them into filtered artificial seawater with clove oil (HUMCO Clove Oil, 1:2500). After anaesthetization, they were transferred into a coated Nunclon Petri dish (60x15mm), where the embryos were gently isolated with the use of fine forceps.

Subsequently, the embryos were kept in Nunclon Petri dishes (35x10mm), 30 embryos in each dish, in filtered artificial seawater supplied with antibiotic (GIBCO® Penicillin/Streptomycin, liquid Cat.No. 15140-122, 1:100) and antifungal (GIBCO® Fungizone® Antimycotic, liquid Cat.No.15290-018, 1:200).

A.4 Live imaging of regeneration

In order to record regeneration, I had to stabilize the amputated, regenerating appendage, while the animal remained alive. Initially, I anaesthetized the animal and amputated the thoracic appendage I wanted to record (usually the fifth thoracic appendage – T5). Subsequently, I glued the amputated appendage, as well as the main body part of the animal, on a cover slip using surgical glue (Dermabond). It is important to glue the animal effectively on the cover slip (otherwise it will escape leaving the amputated appendage on the cover slip) but not too much, because it has to be able to move and feed. The cover slip was mounted in a petri dish filled with artificial seawater. Using this setup, I was able to record images every 10 minutes for up to a week. The animals survive and during the next molt they escape the cover slip.

A.5 Satellite-like cell manipulations

5.1 Transplantations

1. Amputate thoracic appendages (T4-T8) from transgenic animals that express EGFP under the muscle-specific promoter and cut them in small pieces (one podomere per piece).
2. Incubate them in Shields and Sang M3 medium (Sigma-Aldrich) supplemented with 10% foetal bovine serum (Gibco), 0.5 mg/ml gentamycin (Sigma-Aldrich) and 0.25 mg/ml collagenase (Sigma-Aldrich) for 1 hour at room temperature.
3. Rinse the appendages in Shields and Sang M3 medium with 10% foetal bovine serum and 0.5 mg/ml gentamycin. Repeat four times.
4. Using fine forceps, extract disaggregated cells from the appendages. During this step, muscle fibers remain attached to the exoskeleton, while the EGFP-expressing, round satellite-like cells are extracted.
5. Collect 10-15 EGFP-positive satellite-like cells with a microinjection needle.
6. Amputate thoracic appendage (T4 or T5) from a wild-type animal and inject the EGFP-positive satellite-like cells in the area of amputation.

5.2 Satellite-like cell staining

1. Amputate thoracic appendages (T4-T8) from transgenic animals that express EGFP under the muscle-specific promoter and cut them in small pieces (one podomere per piece).
2. Incubate them in Shields and Sang M3 medium (Sigma-Aldrich) supplemented with 10% foetal bovine serum (Gibco), 0.5 mg/ml gentamycin (Sigma-Aldrich) and 0.25 mg/ml collagenase (Sigma-Aldrich) for 1 hour at room temperature.
3. Rinse the appendages in Shields and Sang M3 medium with 10% foetal bovine serum and 0.5 mg/ml gentamycin. Repeat four times.
4. Using fine forceps, extract disaggregated cells from the appendages. During this step, muscle fibers remain attached to the exoskeleton, while the EGFP-expressing, round satellite-like cells are extracted.
5. Collect 50-100 EGFP-positive satellite-like cells with a microinjection needle into an eppendorf.
6. Spin the cells in a Cytospin 4 centrifuge (Thermo Scientific) in 1000 rpm for 5 minutes.
7. Fix cells for 15 minutes in 4% formaldehyde in PTx (1xPBS, 0.1% TritonX).
8. Rinse cells twice with PTx.
9. Wash cells twice with PTx for 10 minutes.
10. Remove PTx carefully and add 150ul of blocking buffer (1x PTx, 5% NGS), incubate for 1 hour.
11. Remove blocking buffer and and add the first antibody solution.
12. Incubate at 4°C overnight.
13. Wash sections with PTx for 5 minutes. Repeat three times.
14. Add the secondary antibody solution.
15. Incubate for 2 hours at room temperature.
16. Wash sections with PTx for 5 minutes. Repeat twice.
17. Add TOPRO or DAPI (if needed). Incubate for 5 minutes.
18. Wash sections with PTx for 5 minutes. Repeat twice.
19. Add 70% glycerol and place the cover slip.

Appendix B

Constructs and primers

Chapter	Purpose	Procedure		Intermediate constructs	Final construct
		Vector	Insert		
1	Ubiquitous constitutive promoter	AscI cut pMi(3xP3-DsRed)	AscI fragment from pECEIII(EF1a-DsRed) sent by Patel		pMi(3xP3-DsRed;EF1a-DsRed)
		pGEM-T easy	PCR amplified U1_SL locus from genomic DNA with primers U1SLFor and U1SLRev	pGEM(U1_SL)	
		NotI/PacI cut pECEIII(Ef1a-DsRed)	NotI/PacI fragment from pGEM(U1_SL)	pECEIII(U1_SL-DsRed)	
		AscI cut pMi(3xP3-EGFP)	AscI fragment from pECEIII(U1_SL-DsRed)		pMi(3xP3-EGFP;U1_SL-DsRed)
		pGEM-T easy	PCR amplified SL_U1 locus from genomic DNA with primers SLU1For and SLU1Rev	pGEM(SL_U1)	
		NotI/PacI cut pECEIII(Ef1a-DsRed)	NotI/PacI fragment from pGEM(SL_U1)	pECEIII(SL_U1-DsRed)	
		AscI cut pMi(3xP3-EGFP)	AscI fragment from pECEIII(SL_U1-DsRed)		pMi(3xP3-EGFP;SL_U1-DsRed)
		pGEM-T easy	PCR amplified SL_SL locus from genomic DNA with primers SLSLFor and SLSLRev	pGEM(SL_SL)	
		NotI/PacI cut pECEIII(Ef1a-DsRed)	NotI/PacI fragment from pGEM(SL_SL)	pECEIII(SL_SL-DsRed)	
		AscI cut pMi(3xP3-EGFP)	AscI fragment from pECEIII(SL_SL-DsRed)		pMi(3xP3-EGFP;SL_SL-DsRed)
		EcoRI/NcoI cut pSL(HS-DsRed)	EcoRI/NcoI cut PCR amplified WSSVie1 promoter from WSSV genomic DNA with primers WSSV-ie1-For and WSSV-ie1-Rev2	pSL(WSSVie1-DsRed)	
		AscI cut pMi(3xP3-EGFP)	AscI fragment from pSL(WSSVie1-DsRed)		pMi(3xP3-EGFP;WSSVie1-DsRed)
		EcoRI/NcoI cut pSL(HS-DsRed)	EcoRI/NcoI cut PCR amplified WSSVie2 promoter from WSSV genomic DNA with primers WSSV-ie2-For and WSSV-ie2-Rev2	pSL(WSSVie2-DsRed)	
		AscI cut pMi(3xP3-EGFP)	AscI fragment from pSL(WSSVie2-DsRed)		pMi(3xP3-EGFP;WSSVie2-DsRed)
	Construct for transcribing Cre	BamHI(blunt)/SgrBI cut pCS(FlpER(T)) sent by Ryffel	SlaI(blunt)/SgrBI fragment from pCre sent by Livet		pCS(Cre)
	Construct for transcribing CreER	BamHI(blunt)/NotI cut pCS(FlpER(T)) sent by Ryffel	EcoRI(blunt)/NotI fragment from pCAG(CreER) sent by Livet		pCS(CreER)
	Lox construct for excision assay	AscI cut pPax sent by MrGene	AscI fragment from pBME by Johannes Schinko	pPax(LoxN-LoxN)	
		HindIII cut pPax(LoxN-LoxN)	HindIII fragment from pMi(3xP3-EGFP; Pax37(intron)-DsRed) (see later)		pPax(LoxN-HindIII fragment-LoxN)
	Cre/lox recombination constructs	NcoI(blunt)/XbaI(blunt) cut pSL(HS-MiTR)	XhoI(blunt)/MluI(blunt) fragment from pCre sent by Livet	pSL(HS-Cre)	
		AscI cut pMi(3xP3-EGFP)	AscI fragment from pSL(HS-Cre)		pMi(3xP3-EGFP;HS-Cre)
		AscI(blunt) cut pMi(AscI)	PacI linkers	pMi(PacI)	
		PacI cut pMi(PacI)	AflII(PacI linker)/NheI(PacI linker) fragment from Brainbow M minus Kusabira by Johannes	pMi(BrMminusKus)	
		BstXI(blunt)/NotI(blunt) cut pSL(HS-DsRed)	PacI(blunt) fragment from pMi(BrMminusKus)	pSL(HS-BrMminusKus)	
		AscI cut pMi(3xP3-DsRed)	AscI fragment from pSL(HS-BrMminusKus)		pMi(3xP3-DsRed;HS-BrMminusKus)

Chapter	Purpose	Procedure		Intermediate constructs	Final construct
		Vector	Insert		
1	**Construct for transcribing Flp**	pGEM-T easy	PCR amplified Flp with primers JS_Flp-BglII-fwd and JS_Flp-StuI-rev	pGEM(Flp)	
		BamHI(blunt)/SgrBI cut pCS(FlpER(T)) sent by Ryffel	NotI(blunt)/SgrBI fragment from pGEM(Flp)		pCS(Flp)
	FRT construct for excision assay	EcoRV/NsiI cut pMi(3xP3-DsRed;HS-Flybow)			pMi(FRT-FRT)
	Flp/FRT recombination constructs	BstXI(blunt)/NotI(blunt) cut pSL(HS-DsRed)	NheI(blunt)/AvrII(blunt) fragment from Flybow2.0 by Salecker	pSL(HS-Flybow)	
		AscI cut pMi(3xP3-DsRed)	AscI fragment from pSL(HS-Flybow)		pMi(3xP3-DsRed;HS-Flybow)
		BstXI(blunt)/NotI(blunt) cut pSL(HS-DsRed)	AatII(blunt)/SstI(blunt) cut pCS(FlpER(T)) sent by Ryffel	pSL(HS-FlpER(T))	
		AscI cut pMi(3xP3-EGFP)	AscI fragment from pSL(HS-FlpER(T))		pMi(3xP3-EGFP;HS-FlpER(T))
		BstXI(blunt)/NotI(blunt) cut pSL(HS-DsRed)	AatII(blunt)/HpaI(blunt) cut pCS(FlpER(T2)) sent by Ryffel	pSL(HS-FlpER(T2))	
		AscI cut pMi(3xP3-EGFP)	AscI fragment from pSL(HS-FlpER(T2))		pMi(3xP3-EGFP;HS-FlpER(T2))
	Construct for creating a stable transposase carrying transgenic line	KpnI cut pBS(MiL;attB;HS-EGFP)#6 by Xaris	-	pBS(HS-EGFP)	
		NcoI/NotI cut pSL(MS-DsRed)	NcoI/NotI fragment from pMi(3xP3-EGFP)	pSL(MS-EGFP)	
		KpnI cut pBS(HS-EGFP)	KpnI fragment from pBS(MiL;attB;HS-EGFP)#6 by Xaris	pBS(attB;MiR;HS-EGFP)	
		SacI/NaeI cut pBS(attB;MiR;HS-EGFP)	SacI/SspI fragment from pMi(3xP3-EGFP)	pBS(attB;MiR;HS-EGFP;MiL)	
		NcoI/NheI cut pBS(attB;MiR;HS-EGFP;MiL)	NcoI/AvrII fragment from pSL(HS-MiTR)	pBS(attB;MiR;HS-MiTR;MiL)	
		AscI cut pBS(attB;MiR;HS-MiTR;MiL)	AscI fragment from pSL(MS-EGFP)		pBS(attB;MiR;HS-MiTR;MiL;MS-EGFP)
	Clonal analysis	NcoI/NotI cut pSL(HS-DsRed)	NcoI/NotI fragment from pMi(3xP3-EGFP)	pSL(HS-EGFP)	
		AscI cut pMi(AscI)	AscI fragment from pSL(HS-EGFP)		pMi(HS-EGFP)

Chapter	Purpose	Procedure		Intermediate constructs	Final construct
		Vector	Insert		
3	**Identification of mesodermal non-muscle cells**	FseI cut pMi(HS-EGFP)	FseI fragment from pSL(MS-DsRed)		pMi(MS-DsRed;HS-EGFP)
	Pax3/7 reporter	NcoI/HindIII cut pPax sent by MrGene	NcoI/HindIII fragment from pSL(MS-DsRed)	pPax(Pax37-DsRed)	
		AscI cut pMi(3xP3-EGFP)	AscI fragment from pPax(Pax37-DsRed)		pMi(3xP3-EGFP;Pax37-DsRed)
		SalI(blunt) cut pMi(3xP3-DsRed)	BamHI(blunt) cut pPax(Pax37-DsRed)	pMi(Pax37-DsRed)	
		AscI cut pMi(Pax37-DsRed)	AscI fragment from pSL(HS-EGFP)		pMi(Pax37-DsRed;HS-EGFP)
		AfeI/NotI cut pPax(Pax37-DsRed)	AfeI/NotI fragment from pSL(HS-DsRed)	pPax(Pax37(intron)-DsRed)	
		AscI cut pMi(3xP3-EGFP)	AscI fragment from pPax(Pax37(intron)-DsRed)		pMi(3xP3-EGFP;Pax37(intron)-DsRed)
		EcoRI(blunt)/EcoRV cut pSL(MS-DsRed)	BamHI(blunt) cut pPax(Pax37-DsRed)	pSL(Pax37(Msintron)-DsRed)	
		AscI cut pMi(3xP3-EGFP)	AscI fragment from pSL(Pax37(Msintron)-DsRed)		pMi(3xP3-EGFP;Pax37(MSintron)-DsRed)
	MS intron activity	EcoRI(blunt)/EcoRV cut pSL(MS-DsRed)		pSL(Msintron-DsRed)	
		AscI cut pMi(3xP3-EGFP)	AscI fragment from pSL(Msintron-DsRed)		pMi(3xP3-EGFP;MSintron-DsRed)

Primer	Sequence	Purpose
U1SLFor	GCGGCCGCGGTAAGTATGCGTTTGCT	amplification of genomic area between U1snRNA and SL
U1SLRev	TTAATTAAGTGAAAATTCAGAGTTGCT	
SLU1For	GCGGCCGCGTGAAAATTCAGAGTTGCT	amplification of genomic area between U1snRNA and SL (reverse orientation)
SLU1Rev	TTAATTAAGGTAAGTATGCGTTTGCT	
SLSLFor	GCGGCCGCGTTTTACTGGTATTGTATCA	amplification of genomic area between two consecutive SL sequences
SLSLRev	TTAATTAAGAAAATTCAAAATGCTTTGCC	
WWSV-ie1-For	CGGAATTCGATGATGGTGATGTTTCTAGG	amplification of WSSV ie1 promoter
WWSV-ie1-Rev2	TTAAAGGCCATGGTGAGTGGAGAGAGAG	
WWSV-ie2-For	CGGAATTCGTCTTCAACATCTTCTTGTTCG	amplification of WSSV ie2 promoter
WWSV-ie2-Rev2	ATCGCCATGGTGAAGATCTCTGGGAAATG	
CreAsFOR	CAGGGTTTTCCCAGTCACG	primers for excision assay for testing the activity of Cre
CreAsREV	CAGCGAGTCAGTGAGCGAG	
FlpAsFor	GTGTAATGGCTTTCGGCAGT	primers for excision assay for testing the activity of Flp
FlpAsRev2	TTGGAAAGATTCGGAGTTCG	
Pax3/7-2PCRF	ATGTCGTCTGCAGGACAAGGC	*Ph*Pax3/7-2 probe preparation
Pax3/7-2PCRR	GAGGCAGCTGAGGGTGTCC	
JS_Flp-BglII-fwd	ATAGATCTATGCCACAATTTGATATATTATG	subcloning of Flp ORF in pGEM-T easy
JS_Flp-StuI-rev	TAAGGCCTTATATGCGTCTATTTATGTAGGATG	

Appendix C

Literature

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