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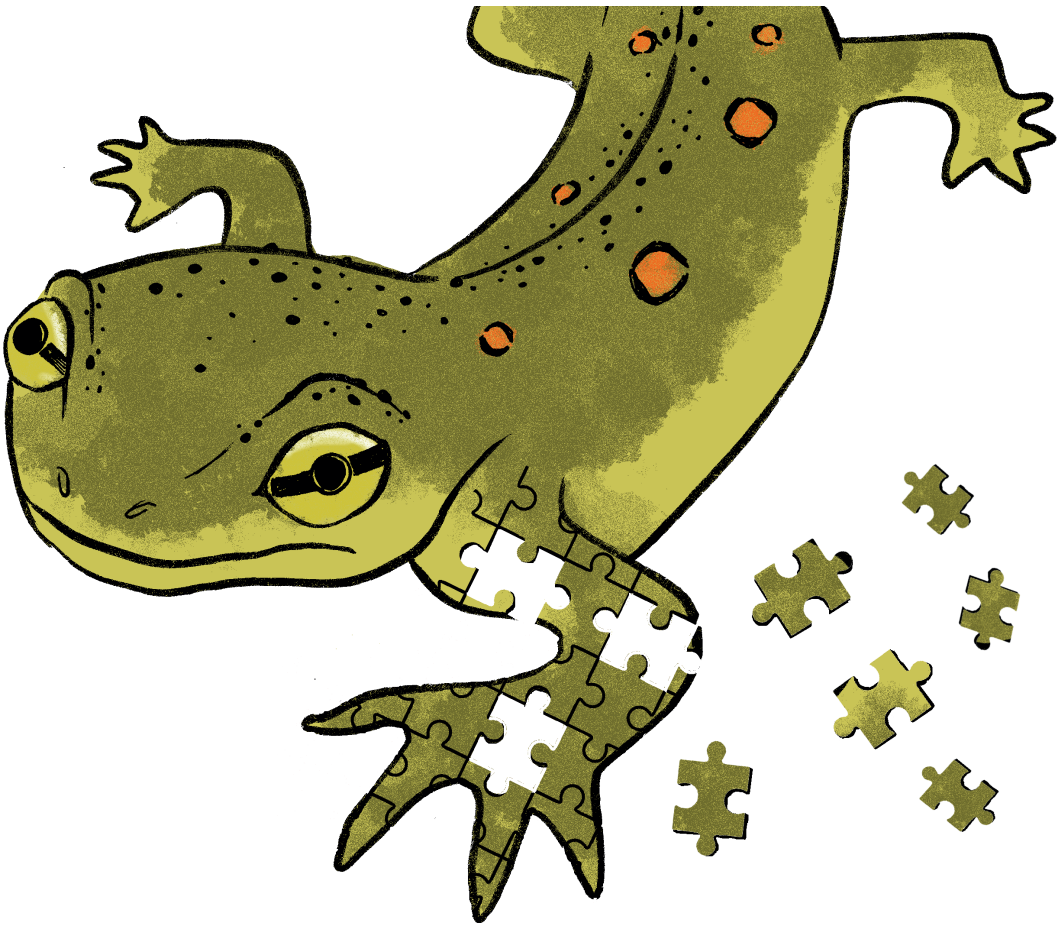
Molecular Mechanisms of Salamander Limb Regeneration



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Molecular Mechanisms of Salamander Limb Regeneration

THESIS FOR DOCTORAL DEGREE (PH.D.)

By

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To my family

"The world is full of wonders, but they become more wonderful,
not less wonderful, when science looks at them."

David Attenborough

Abstract

Salamanders, like newts and axolotls, stand out among adult vertebrates for their outstanding capacity to regenerate whole body parts and restore complex structures upon injury. One of the best-known examples is their ability to fully regenerate a functional limb. Despite the important progress in the field, our understanding of the molecular cues that control limb regeneration is still limited. In this thesis, I focus on the mechanisms by which skeletal muscle stimulates limb regeneration. Skeletal muscle is particularly interesting because, in newts, it contributes to limb regeneration by dedifferentiation. This unique process is characterized by fragmentation of the multinucleated myofiber and subsequent cell cycle reentry by the derived mononucleate progeny.

In **Paper I**, we sequenced and edited the ~20 Gigabases genome of the Iberian ribbed newt *Pleurodeles waltl*, a commonly used species for regeneration studies in salamanders. Using CRISPR/Cas9 technology we perturbed two key transcription factors (Pax3 and Pax7) that are involved in skeletal muscle development and regeneration in vertebrates. We found that contrary to mammals, in which Pax7 expression by skeletal muscle stem cells is indispensable for regeneration, muscle regeneration was not altered when Pax7 gene was mutated in newts. Moreover, we observed that embryonic stem cell-specific microRNAs (mir-93b and mir-427), as well as Harbinger DNA transposons carrying the Myb-like proto-oncogene have expanded dramatically in the *Pleurodeles waltl* genome and are co-expressed during limb regeneration. This study provides a foundation for comparative genomic studies that could improve our understanding of the uneven distribution of regenerative capacities among vertebrates.

In **Paper II**, we identified a microRNA, miR-10b-5p, which is highly abundant in muscle tissue across species and downregulated during early limb regeneration in newts. In contrast, miR-10b-5p displayed the opposite regulation in mammalian cultured myotubes, when these were induced to dedifferentiate. To investigate a possible function of miR-10b-5p in newt limb regeneration, we overexpressed it by mimic injection. We found that such manipulation of miR-10b-5p levels during the initial stages of regeneration slowed down the regeneration process. Moreover, we observed that overexpression of miR-10b-5p decreased the number of cycling cells and counteracted blastema growth. The identification of miR-10b-5p targets will be an important task for future studies.

In **Paper III**, we showed that blood clotting proteases cleaved and activated blood-derived bone morphogenetic proteins (BMPs) to promote BMP signaling-dependent cell cycle re-entry by myofiber progeny. In particular, we found that protease-activated BMP4/7 heterodimers which were present in serum, strongly induced myotube cell cycle re-entry, with protease cleavage yielding a 30-fold potency increase of BMP4/7 compared with canonical BMP4/7. Additionally, we observed that inhibition of BMP signaling, via muscle-specific dominant-negative receptor expression, reduced cell cycle re-entry *in vitro* and *in vivo*. Furthermore, *in vivo* inhibition of serine protease activity depressed cell cycle re-entry, which in turn could be rescued by cleaved-mimic BMP. This work provides a new molecular mechanism for the reversal of the differentiated state in muscle.

In **Paper IV**, we carried out a comparative analysis of centrosome dynamics in mouse and newt muscle cells. We showed, through a detailed characterization of different centrosome components, that centrosomes were gradually disassembled during muscle differentiation in mammals. We also provided new insights into the underlying mechanisms and variations in gene expression during that inactivation process. On the other hand, we found that salamanders retained several centrosome components even in mature myofibers. Moreover, we observed that not only the centrosomes were maintained in salamander muscle, but they also appeared to be active as microtubule organizing centers. This study has elucidated fundamental differences between vertebrates at cellular level, which might help us to understand why species differ in their ability to produce regenerative progenitor cells.

List of scientific papers

I. Reading and editing the *Pleurodeles waltl* genome reveals novel features of tetrapod regeneration

Ahmed Elewa, Heng Wang, Carlos Talavera-López, Alberto Joven, Gonçalo Brito, Anoop Kumar, L. Shahul Hameed, May Penrad-Mobayed, Zeyu Yao, Neda Zamani, Yamen Abbas, Ilgar Abdullayev, Rickard Sandberg, Manfred Grabherr, Björn Andersson, András Simon

Nature Communications (2017), 8, 2286

II. Deregulation of miR-10b-5p impairs early-stage events during newt limb regeneration

Gonçalo Brito, Anoop Kumar, Elaiya Raja Subramanian, Ahmed Elewa, Karen Echeverri, András Simon

Manuscript

III. Serum proteases potentiate BMP-induced cell cycle re-entry of dedifferentiating muscle cells during newt limb regeneration

Ines Wagner, Heng Wang, Philipp M. Weissert, Werner L. Straube, Anna Shevchenko, Marc Gentzel, Gonçalo Brito, Akira Tazaki, Catarina Oliveira, Takuji Sugiura, Andrej Shevchenko, András Simon, David N. Drechsel and Elly M. Tanaka

Developmental Cell (2017), 40, 608–617

IV. Key differences in the regulation of microtubule organizing centers comparing mammalian and newt muscle cells

Gonçalo Brito, Mariana Lince-Faria, Mafalda Pimentel, Anoop Kumar, Ksenia Volkova, Elaiya Raja Subramanian, Edgar Gomes, András Simon, Mónica Bettencourt-Dias

Manuscript

Contents

List of abbreviations

AEC	Apical epidermal cap
AER	Apical ectodermal ridge
CNTB	Centrobin
DGCR8	DiGeorge syndrome critical region 8
Dpa	Days post-amputation
DRG	Dorsal root ganglia
ECM	Extracellular matrix
Gb	Gigabases
Ggf2	Glial growth factor 2
LINE-1	Long interspersed nucleotide element- 1
lncRNA	long non-coding RNA
LTR	Long terminal repeat (retrotransposon)
miRISC	miRNA-induced silencing complex
miRNA	MicroRNA
MLP	MARCKS-like protein
MN	Myonuclei
mRNA	Messenger RNA
MTOC	Microtubule organizing center
nAG	Newt anterior gradient (protein)
ncRNA	Non-coding RNA
NRG1	Neuregulin-1
Nts	Nucleotides
PCD	Programmed cell death (PCD)
PCM	Pericentriolar material
PCNT	Pericentrin
PD	Proximo-distal (axis)
PECs	Pigment epithelial cells
piRNA	PIWI-interacting RNA

Plk1	Polo-like kinase 1
Rb	Retinoblastoma
RISC	RNA-induced silencing complex
RNA-seq	RNA-sequencing
rRNA	Ribosomal RNA
Sas4	Spindle assembly abnormal 4
SC	Satellite cells
siRNA	small interfering RNA
sncRNA	Short non-coding RNA
snRNA	small nuclear RNA
snoRNA	small nucleolar RNA
TE	Transposable elements
tRNA	Transfer RNA
UTR	Untranslated region
γ-tub	Gamma-tubulin

1. Introduction

Regeneration stands as one of the most fascinating phenomena in biology. Usually defined as the capacity of an organism to restore body parts that have been damaged or lost, it is a widespread feature throughout the animal kingdom, but the extent of regenerative responses varies considerably between organisms (Carlson, 2007). Humans, for instance, are not particularly good in repairing tissues and organs that have been affected by age, disease or trauma (Jaźwińska and Sallin, 2015), which raises an important question. Is this the result of an irreversible loss of regenerative abilities during mammalian evolution? Or is this due to acquired mechanisms that might suppress a latent regenerative capacity, thus having the potential to be unlocked? While arguments have been presented for both models over the years, the discussion could not be settled. A better understanding of the molecular mechanisms controlling regeneration should provide new perspectives to revisit this evolutionary question.

The discovery of stem cells, which are present in many body tissues and can be recruited to repair damage (Blau, Brazelton and Weimann, 2001), has been particularly influential in the recent decades and revived the interest in human regeneration. Their self-replicating and differentiation potential, makes them attractive candidates for new regenerative medicine therapies. However, despite holding great promise, the success of cell-based therapies has been relatively limited so far (Ankrum and Karp, 2010; Buzhor *et al.*, 2014). In part, this reflects how we currently lack a complete understanding of how these cells are implicated in tissue growth, maintenance and regeneration. This has not affected the general ambition of enhancing regeneration of human body parts, but the reality highlights the need to take a step back and focus on expanding our knowledge regarding the basic principles that guide tissue regeneration.

In order to do so, one of the best tools at our disposal is the study of other organisms with high regenerative capacity, which can contribute with important new insights about regulatory mechanisms at the cellular and molecular level. In particular, it is fundamental to understand what prevents different animals from regenerating similar body structures. What are the roadblocks to human regeneration and what allows regeneration in other organisms? Among vertebrates, salamanders, such as newts, have been instrumental in many of the fundamental discoveries made in regenerative biology (Brockes and Kumar, 2008). They stand out for their exceptional regenerative capabilities and are well-known for restoring full limbs upon amputation (Stocum, 2017). The limb provides a particularly good system to study the regeneration process, as it is easily accessible for experimental manipulation and allows for regeneration to be monitored in a non-invasive fashion.

The process of salamander limb regeneration (Fig. 1) has been the central theme of this doctoral thesis, with a particular focus on the role of skeletal muscle and the events of muscle dedifferentiation. The following chapters are intended to provide the necessary background and the appropriate context to the different projects that constitute this work. Considering the broad range of topics described, I will, in the interest of clarity, only cover the aspects of each subject that I consider to be more relevant.

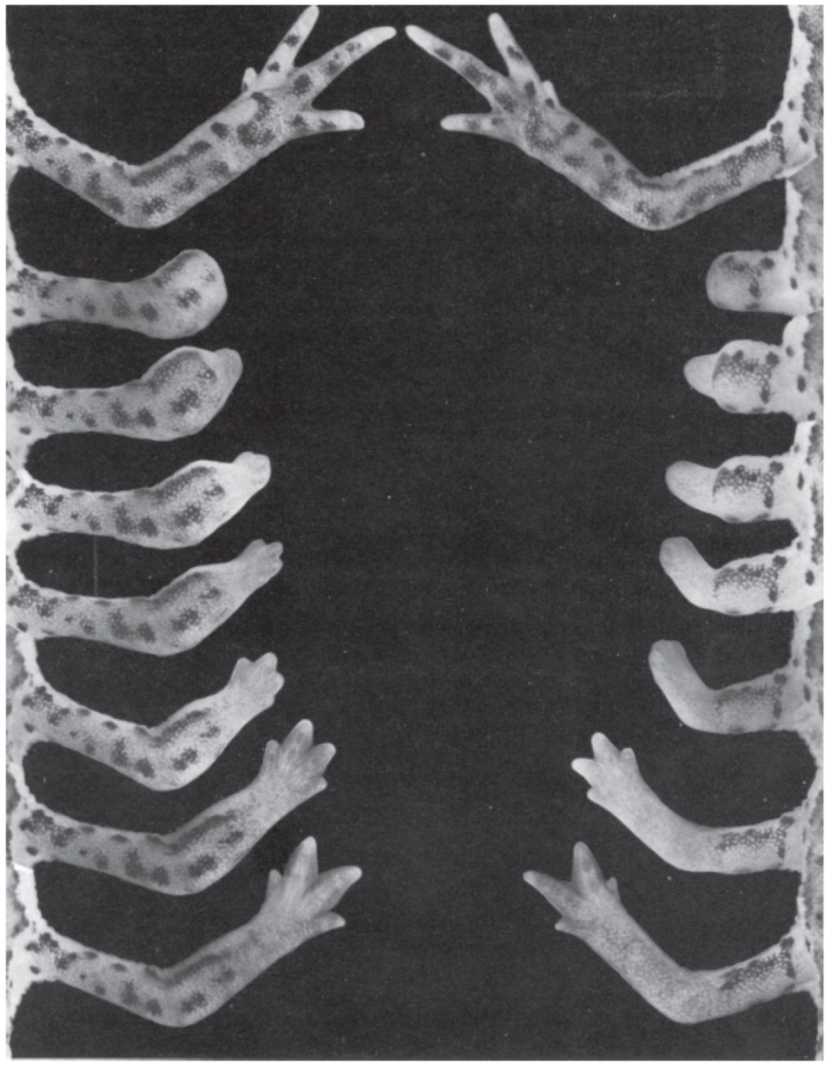

Figure 1: Successive stages in the regeneration of newt limbs following amputation at proximal (right) and distal (left) levels. At the top are the original limbs, while below the intervals of regeneration are 7, 21, 25, 28, 32, 42, and 70 days after amputation. From proximal amputation, the limb elongates faster but differentiates slower than the limbs regenerating from a more distal level (Reproduced with permission from: Goss R.J. 1969).

2. The study of regeneration

Despite being an old research field, the study of regeneration has progressed slowly for a long period. The lack of suitable techniques to dissect such a high degree of complexity has been a major obstacle to answer the prevailing questions. For most of its existence, regeneration research has been heavily dependent on morphological observations and grafting experiments, however, the expansion of molecular biology completely changed the landscape of the regenerative biology. With the constant development of new genetic tools, it has become possible to label and trace specific cells and tissues, which has been crucial to address questions of tissue origin and changes in the differentiation state of cells. In this chapter, I will provide an overview of the field, outlining how the initial curiosity in the natural phenomenon of regeneration led to the creation of a new scientific discipline, and elaborate on some of its major discoveries. I will then proceed by exploring what is known to date regarding mammalian and salamander regeneration. Lastly, I will conclude by describing the progress in regenerative medicine and how the study of regeneration has influenced the development of new therapies.

2.1 An historical perspective

The phenomenon of regeneration has been known since the ancient civilizations, when it was observed that lizards could regrow their tails, as described by Aristotle (Aristotle and Barnes, 2014). Unsurprisingly, we can find several references to regenerative events in Greek mythology such as the legend of Prometheus who, in a punishment ordered by Zeus, was chained to a rock, where every day an eagle would come to feed on his liver, while every night the liver regenerated to its original form. Another example is the more popular legend of Heracles (or Hercules) and the Hydra, a serpent-like creature with multiple heads, that would regrow its heads every time they were severed by Heracles' sword (Dinsmore, 1991; Graves, 2017).

The first scientific demonstration, however, was only reported in 1686 when Melchisedech Thevenot presented the case of lizard tail regeneration to the Paris Academy of Science, with a series of observations confirming those already made by Aristotle two thousand years before. Later on, already in 1712, animal regeneration was revisited by René-Antoine Réaumur, a French naturalist who reported that crayfish could loose and regrow their appendages (Dinsmore, 1996). It is worth mentioning that, at the time, the concept of preformation was believed to be behind such regenerative events, in which regeneration would result from the expansion or unfolding of very small limbs already preformed at the base of the existent limb (Carlson, 2007). These earlier results were received with great excitement and scientists became eager to discover other examples throughout the animal kingdom. This was finally achieved in 1744, when Abraham Trembley reported that aquatic polyps or hydras, named for their resemblance to the famous mythological creature, also displayed regenerative abilities, thus confirming a more prevalent phenomenon in nature. But the impact of Trembley's discovery was more than simply adding a new entry to the list of regenerative organisms. His observations strongly argued against the idea of preformation, since he observed that two full hydras would originate from one transected animal (Fig. 2). This had profound implications in the scientific and philosophical debate of the time and this disruptive context ultimately gave rise to the foundations of experimental zoology and more specifically to the discipline of developmental biology (Dinsmore, 1991, 1996; Leclère and Röttinger, 2017).

The movement kept growing rapidly as similar observations were recorded in other studies using annelid worms, by C. Bonnet in 1745, snails, frog tadpoles and adult salamanders, by L. Spallanzani in 1768, and planarians by P. S. Pallas in 1774. The pioneer studies of Spallanzani, where he thoroughly characterized the regeneration of salamander tails, limbs and jaws, were particularly important because they placed vertebrate regeneration under the spotlight, unlike the lizard tail observations that

remained unnoticed by many in the scientific community. The fact that regeneration was not an exclusive feature of invertebrates and could also be seen in organisms of higher complexity was a turning point and attracted the interest of more scientists towards the study of this phenomenon. Additionally, Spallanzani's work greatly contributed to the beginning of a gradual shift from scientific studies that were traditionally driven by highly descriptive observations towards a more experimental and hypothesis-driven approach to study nature (Dinsmore, 1996; Carlson, 2007).

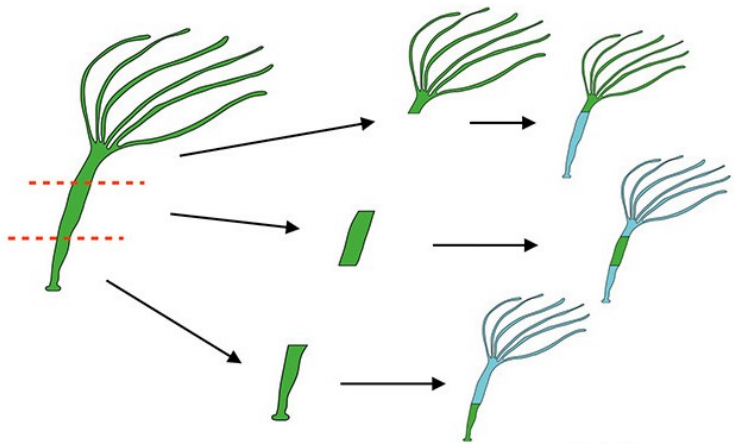

Figure 2: Regeneration of the fresh water polyp (*Hydra*). Upon amputation, each fragment will give rise to a new animal by regenerating the missing body parts. (From: Leclère and Röttinger, 2017)

During the 19th century, and before the era of genetics, two key events took place that changed the understanding of biology. First, the formulation of the cell theory in 1839 by the joint work of Schleiden and Schwann, which postulated that the cell was the basic unit of both plants and animals (Ribatti, 2018). This new understanding of tissue composition stimulated the development of histological techniques, which became very powerful tools in regeneration research. Second, the theory of heredity proposed by August Weismann in 1892, which was based on the concept of the germ-plasm, an hereditary substance contained in the germ cells that carries information to the offspring (Churchill, 1968; Zou, 2015). Inspired by previous work from other biologists such as Charles Darwin and Alfred Wallace, which independently formulated the theory of evolution through natural selection in 1858 (Kutschera and Niklas, 2004), Weismann's controversial hypothesis expanded this concept and attempted to explain simultaneously diverse biological phenomenon such as development, regeneration and evolution.

In the same period, Thomas Morgan, who later became famous for his studies on *Drosophila* genetics, also dedicated part of his career to the study of regeneration. His initial motivation was to understand differentiation in development, which he found difficult to comprehend simply by studying developing embryos (Sunderland, 2010). As regeneration was thought, by some, to mimic the basic processes of development, Morgan decided to use regeneration experiments to find the answers he was seeking and so he directed his efforts towards the study of the regenerative abilities of planarians (Morgan, 1898, 1901a) among other organisms. In order to better distinguish between the diverse cases of regeneration, he became committed to define a consistent nomenclature to be used in the classification of regenerative phenomena. In his book *Regeneration* (Morgan, 1901b), he subdivided regeneration in two different categories, which are still relevant today: "epimorphosis", when the development of the new part is driven by cellular proliferation, and "morphallaxis", which results from the reorganization of the existent material without proliferation. Additionally, in the same publication Morgan provided his main contribution to the field, by demonstrating that regeneration is a general feature of the organism's growth and thus is relevant for the understanding of development. This was against Weismann's view that regeneration should be studied as an adaptation and a product of natural selection. Despite his important contribution to regeneration studies, it is reported that Morgan eventually changed his focus to the emerging field of genetics because he believed regeneration was far too complex to be solved during his lifetime (Carlson, 2007). Interestingly, his predictions were not entirely unfounded as many of the questions he wondered about still remain unanswered today.

During the 20th century and with the progress of histology, researchers started to describe in detail several regenerative processes in different species. Nevertheless, due to the apparent lack of capacity for many human tissues to regenerate, the interest in mammalian regeneration started to decrease gradually. In contrast, amphibian limb regeneration received special attention with several scientists shifting from pure observations towards experimental studies, to investigate the role of specific tissues in limb regeneration – Given its importance for this thesis, the work conducted during this period will be discussed later in greater detail. The field gained new momentum at the turn of the century with the expansion of stem cell research, after scientists identified stem cells in the adult body (Blau, Brazelton and Weimann, 2001) and realized the potential of for human tissue regeneration (Gage, 2000; Jankowski, Deasy and Huard, 2002). There are still high expectations for the use of stem cells today, but even though we witnessed an increasing number of tools for cell replacement therapies, our ability to functionally replace lost tissues is still limited and requires further research (Fox *et al.*, 2014; Heslop *et al.*, 2015).

2.2 Diversity of regenerative phenomena

Although regeneration is generally defined as the capacity of an organism to restore tissues or organs that have been lost or damaged (Sánchez Alvarado and Tsonis, 2006; Brockes and Kumar, 2008), this has always been a matter of debate and not everyone agrees on a single definition. Regenerative biology is a broad research field and one of its main challenges comes from the fact that not all animals can regenerate body parts but also that not all the tissues within the body share the same regenerative potential (Goss, 1969; Tsonis, 2000). Over the years, the increasing number of regenerative phenomena being described led to many discussions on how to create a classification system that could integrate all the examples into different types of regeneration. However, even today it is still hard to fit some of the known examples into specific categories, either because they are poorly understood or because of their unique features. A good example is the case of lens regeneration in newts which, in spite of being a well-studied phenomenon, has not been assigned to a particular type of regeneration. Regeneration phenomena can be grouped into four major types: Physiological regeneration, Reparative regeneration, Hypertrophy and Morphallaxis (Fig. 3) (Carlson, 2007).

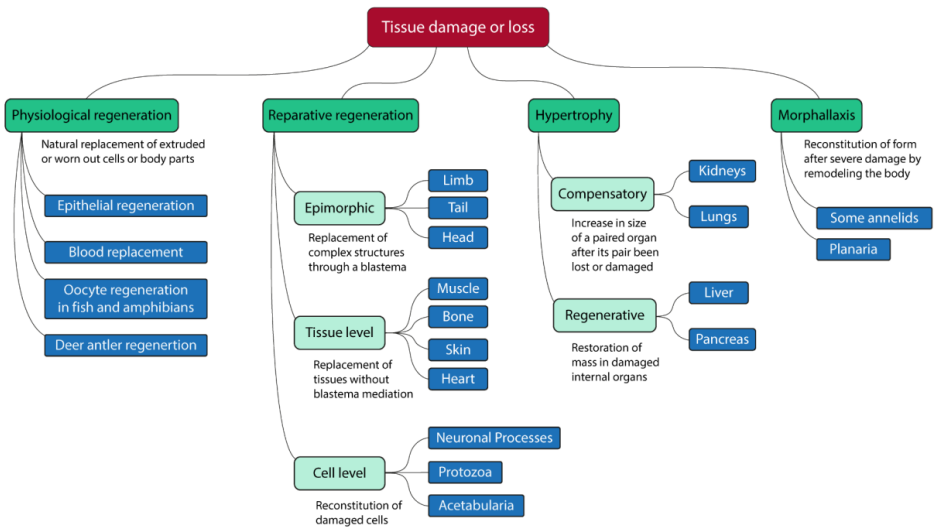

Figure 3: Classification of the major types of regenerative phenomena present in the animal kingdom. In this classification they are divided between four main groups: Physiological regeneration, Reparative regeneration, Hypertrophy and Morphallaxis (Based on: Carlson, 2007).

Physiological regeneration is the term used to describe the cyclical replacement of worn-out body parts during homeostasis in which older cells are eliminated and replaced by new cells. Mammals display various examples of physiological regeneration such as the renewal of the skin epidermis (Blanpain and Fuchs, 2009) and blood cells (Eaves, 2015), but perhaps the most remarkable example is the annual regeneration of deer antlers (Goss, 1983). The latter is a stem-cell based process that results from the recruitment, proliferation and differentiation of local stem cells and constitutes the only known case of mammalian full appendage regeneration (Kierdorf and Kierdorf, 2011). Although these examples are grouped under the same category due to their cyclic nature, the difference in complexity is striking, showing that this designation does not imply a particular mechanism shared between the distinct processes. Instead, the term includes a variety of events that help mediating the normal equilibrium of different tissues in the body.

Reparative regeneration refers to the repair of damaged cells, tissues or more complex structures like entire organs or multi-tissue body parts. This category also includes a multitude of underlying mechanisms, but in common is the context of replacement of a structure that was lost or damaged. An example of reparative regeneration at the tissue level is the repair of mammalian muscle (Dumont *et al.*, 2015). When reparative regeneration regards more complex structures, it is called epimorphic, following the term coined by T. H. Morgan, but its definition has changed over time. Currently, epimorphic regeneration refers to the phenomena that involve the formation of a blastema, a mass of undifferentiated cells that gives rise to the regenerate. This is the definition used today by most authors and the one that will be taken into account throughout this thesis. Two of the best examples in vertebrates are limb regeneration in salamanders (Fig. 1) and fin regeneration in teleost fish (Fig. 4) (Brockes and Kumar, 2008; Pfefferli and Jaźwińska, 2015). The former will be explored in detail later in this chapter.

Hypertrophy is the increase in mass of certain internal organs to compensate a missing part, such as the liver (Michalopoulos, 2013), or a missing pair organ, like the kidneys (Addis and Lew, 1940). Here, the focus is on restoring the mass in order to regain its functionality, rather than recovering the original anatomical structure. In opposition to

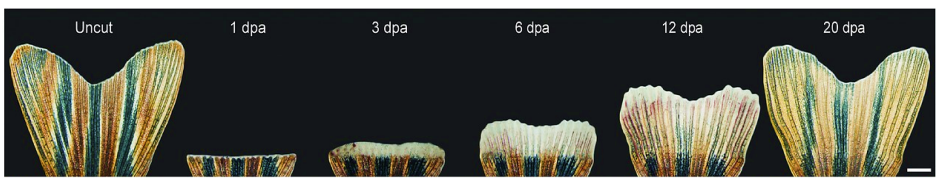

Figure 4: Caudal fin regeneration in Zebrafish. The original fin (Uncut) presents a bi-lobed morphology, which is restored after 20 days post-amputation (dpa). The blastema, a mass of undifferentiated cells that contributes to the new tissue forms between 1 and 3 dpa. (From: Pfefferli and Jaźwińska, 2015)

epimorphic regeneration, where the regenerative response occurs mostly at the amputation site, in hypertrophy the regeneration events occur in the whole remainder of the organ. Interestingly, most of the organs with such capacity can also display it in contexts where there is a higher functional demand, even in the absence of injury or organ removal (Goss, 1966). Liver regeneration after partial hepatectomy provides one of the most-studied examples of hypertrophy, with most studies using the model of surgical removal of two-thirds of the liver in rodents. First proposed by Higgins and Anderson in 1931, this model received a lot of interest with the increasing number of studies from transplanted human livers. However, it should be noted that mammalian livers generally regenerate through cell proliferation, with each cell type replacing its own cell compartment, rather than through an increase in cell size, so the term hyperplasia would be more appropriate to classify this process (Michalopoulos, 2013). Interestingly, when cell proliferation fails, liver regeneration can still occur through transdifferentiation events, such as hepatocytes transdifferentiating into biliary epithelial cells and vice-versa (Michalopoulos, 2011).

Morphallaxis, concerns the reconstruction of the body after severe damage through remodeling events. This type of regeneration can be observed in *Hydra* and *Planaria* (Pellettieri, 2018). Even though planarians can also restore lost organs through epimorphic regeneration, when the body axes are disrupted by injury, the remaining tissues must reset their positional identities and redefine them according to the new anatomical locations (Fig. 5). For example, when only a small head fragment is left, part of those tissues that had an anterior identity will acquire posterior features.

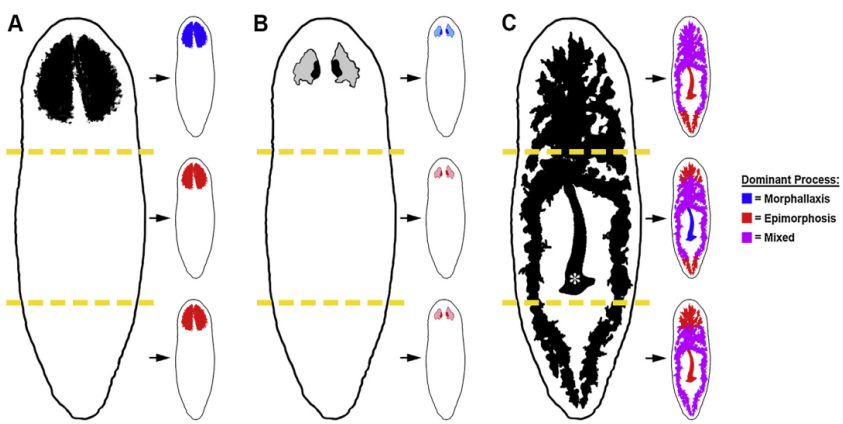

Figure 5: Schematic illustration of morphallactic and epimorphic regeneration in planarians. Head fragments reduce the size of the existing brain (A) and photoreceptors (B) through morphallaxis (blue), whereas trunk and tail fragments form these organs *de novo* in the blastema (a predominantly epimorphic response; red). Regeneration of the gut and pharynx (C) requires the combined action of both processes. (Reproduced with permission from: Pellettieri, 2018)

2.3 Regeneration in mammals

Accumulating observations have shown that adult mammalian tissues harbor significant potential for flexibility and plasticity (Wagers and Weissman, 2004). First, it was established that the differentiated state of cells is not irreversible, but rather continuously maintained, and thus has the potential to be reverted (Blau *et al.*, 1985). Second, resident cells with stem cell properties or potential were identified in a large spectrum of tissues (Tsai, Kittappa and McKay, 2002). Third, nuclear transfer and forced expression of defined factors show that the nuclei from adult cells can reconstitute an entire organism following appropriate manipulations (Gurdon, 1962; Takahashi *et al.*, 2007). Nevertheless, mammalian vertebrates display very limited regeneration abilities in adult life including: renewal of blood cells, epithelium of skin and gut, replacement of muscle and bone tissue and compensatory growth of liver tissue (Odelberg, 2005; Godwin, 2014a).

In the event of an organ loss or severe injury, animals generally respond in two ways: regeneration or repair. Whereas regeneration restores both the integrity and functionality of the tissue, repair usually involves the formation of a scar that will seal the wound with no or partial replacement of the missing tissue (Jaźwińska and Sallin, 2015). Mammals generally respond to severe injuries with high deposition of extracellular matrix, characteristic of the fibrotic scar, which alters the organ structure and impairs its function (Zeisberg and Kalluri, 2013). Hence, the fibrotic process seems to be a major obstacle that prevents a regenerative response in mammals, suggesting a mutually exclusive relationship between regeneration and scar-formation (Singh *et al.*, 2015). Interestingly, scar-free healing and regeneration of several tissues is consistently observed in mammals at early developmental stages, such as in the case of the neonatal mouse heart (Porrello and Olson, 2014), but is gradually lost during development (Godwin, 2014b).

There are, however, few examples of mammals that found ways of bypassing these limitations during adulthood. One classic example is the already mentioned regeneration of deer antlers. Another recently discovered example is the case of the African spiny mouse, an emerging model for regeneration studies, which has the capacity to shed and subsequently regenerate big portions of its skin, a response that might have evolved to escape predators (Seifert *et al.*, 2012). In addition, this study became the first reported case of mammalian autotomy and revealed the capacity of these mice to regenerate hair follicles, sebaceous glands, dermis and cartilage in a scar-free fashion. This highlights the importance of expanding our views towards unconventional model organisms. In particular, they might bring us closer to understand whether the limited regenerative response found in mammals results from critical molecular processes being no longer active, or if they are actively blocked in many adult mammalian tissues.

Considering that regeneration is widespread in the animal kingdom but without being present in all phyla, its origin has been at the center of one of the oldest debates in the field. Nevertheless, it remains unclear whether regeneration emerged independently during evolution or if it represents a common ancestral feature, which has been gradually lost over time in certain species (Brockes and Kumar, 2008; Bely and Nyberg, 2010). New evolutionary studies addressing the intricate relationships between different species are necessary to shine some light on this complex question.

In humans, regenerating a full limb is still regarded by some as the Holy Grail of regenerative medicine, but different attempts to achieve this have been largely unsuccessful. This reflects to some extent the biological constraints of the mammalian system, the current limitations in the field of tissue engineering (see section 2.5), but also our poor understanding of the full complexity of regeneration and its molecular regulation (Ricci, 2013; See, Kulkarni and Pandit, 2013; Shieh and Cheng, 2015; Quijano *et al.*, 2016). Therefore, it is essential that we learn more about regeneration-competent organisms and salamanders are one example that can provide us with new insights regarding key mechanisms that allow regeneration to take place.

2.4 Regeneration in salamanders

In contrast to mammals, some other vertebrates retain the exceptional ability to regenerate complex body parts throughout life. Among them, the most studied examples are the teleost fish, such as the zebrafish, and the aquatic salamanders, such as newts and axolotls (Brockes and Kumar, 2008). Research using zebrafish as a model organism has demonstrated the extensive capacity of this bony fish to regenerate several structures, like the fins (Fig. 4) (Akimenko *et al.*, 1995; Pfefferli and Jaźwińska, 2015), the spinal cord (Becker *et al.*, 1997), the retina (Vihtelic and Hyde, 2000) and the heart (Poss, Wilson and Keating, 2002). Salamanders, however, are regarded as the animals with the largest repertoire of structures that can be regenerated upon injury, which includes: jaws (Goss and Stagg, 1958; Ghosh, Thorogood and Ferretti, 1994), lens (Reyer, 1954; Tsonis, Madhavan, Tancous, *et al.*, 2004), retina (Young, 1967; Mitashov, 1996), heart (Oberpriller and Oberpriller, 1974; Neff, Dent and Armstrong, 1996), tail and spinal cord (Holtzer, 1956; Diaz Quiroz and Echeverri, 2013), and limbs (Dinsmore, 1996; Stocum and Crawford, 2015). Among these regenerative phenomena, the case of limb regeneration in salamanders is one of the oldest being studied and remains as one of the most fascinating.

2.4.1 Limb regeneration

Limb regeneration in salamanders is a complex process that includes several morphological stages, but is generally characterized by three main events. Upon amputation, the stump tissue responds to the injury by a rapid wound healing phase, in which epithelial cells cover the exposed tissue, forming the wound epidermis. This provides important signals that will induce the formation of the blastema, a heterogeneous mass of mesenchymal cells that forms at the wound site originating from the stump tissues. These mesenchymal cells will then proliferate, re-differentiate and proceed to form the new appendage through morphogenesis (Fig. 6) (Iten and Bryant, 1973; Brockes and Kumar, 2002). This represents a classical example of epimorphic regeneration, as the regenerative response is mediated by a blastema (Carlson, 2007).

Θ The wound epidermis and the apical epidermal cap

The wound epidermis, which starts assembling immediately after amputation, is formed through the migration of epidermal basal cells. While the migrating cells do not proliferate (Hay and Fischman, 1961), a group of dividing epidermal cells located proximal to the wound area, provides a continuous stream of migrating cells (Lash, 1955; Repesh and Oberpriller, 1978, 1980). As the blastema cells accumulate, a thickening of the epidermis

can be observed, which will then allow the formation of the apical epidermal cap (AEC). The AEC is an analogous structure to the apical ectodermal ridge (AER) of amniote developing limbs, which functions as a distal signaling center that stimulates blastema proliferation (Stocum, 2017). The importance of the wound epidermis has been well documented in early studies, showing that removal or mechanical disruption of this structure can prevent regeneration (Goss, 1969).

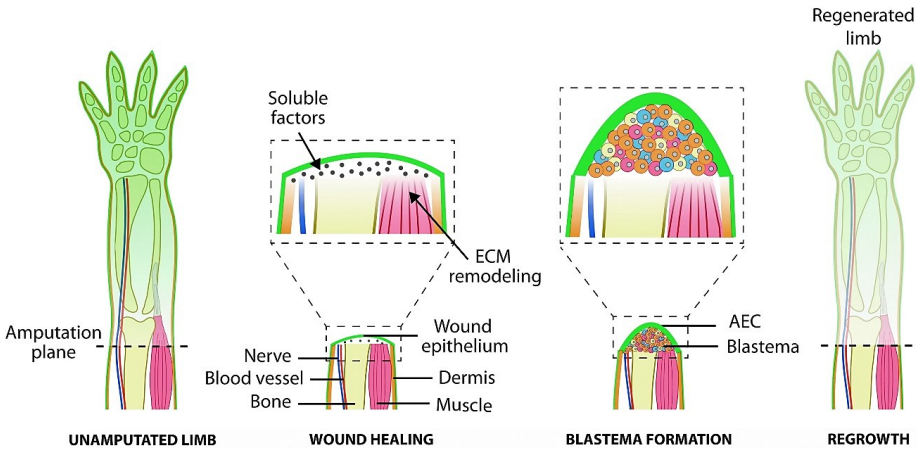

Figure 6: Main events during salamander limb regeneration. An intact limb consists of tissues of various types, including dermal, skeletal, neural, and vascular. After amputation, the wound heals to form an epidermal layer, the underlying tissues undergo remodeling of the extracellular matrix (ECM), and cells in the region secrete soluble factors. Upon thickening of the wound epidermis, the apical epidermal cap is generated, which will send stimulating signals for blastema formation. The blastema consists of a heterogeneous cell mass that originates through proliferation and migration of cells from the adjacent tissues. The blastema then gives rise to the various new tissues that are spatially patterned to reconstruct the original limb structure. (Reproduced with permission from: Quijano et al., 2016)

Θ The origin of the limb blastema

The origin of the limb blastema has been a long-standing question in Regenerative Biology research. Two possible sources have been considered, proposing that either it is generated from the activation of resident stem cells or through the dedifferentiation of mature cells (Simon and Tanaka, 2013; Stocum, 2017). The blastema has long been defined as a dedifferentiation product, however, it is fundamental to distinguish between tissue- and cell-level dedifferentiation to better understand this question. While it was established that tissue dedifferentiation takes place, as it can be observed by the disorganization of the tissue structure upon damage (Iten and Bryant, 1973), it had long remained unresolved whether mature cells generate the pool of undifferentiated cells by reverting their differentiated state and re-entering the cell cycle (Brockes and Kumar,

2002). Several studies have demonstrated that the blastema originates from the mesodermal tissues subjacent to the wound epidermis (Butler and O'Brien, 1942; Thornton, 1942), however, it is essential to consider how the different tissue types contribute to the regenerated limb.

The wound epidermis, despite being critical for regeneration to occur, was shown not to contribute for the blastema (Riddiford, 1960). Another study found that dermal fibroblast-derived cells account for nearly half of the blastema population, whereas the percentage of cartilage-derive cells was relatively low (Muneoka, Fox and Bryant, 1986). Other reports found similar results in relation to the skeletal elements (cartilage/bone), supporting a small or no contribution to the blastema (Steen, 1968; McCusker *et al.*, 2016). In fact, when the bone was removed from the limb prior to amputation, the limbs regenerated all the necessary tissues, including skeleton, suggesting that these tissues are not required for limb regeneration to occur (Thornton, 1938b; Goss, 1956). In contrast, other studies reported that cartilage grafts into irradiated limbs could lead to limb regeneration and contained different tissues, proposing that these derived from chondrocyte dedifferentiation (Eggert, 1966; Wallace, Maden and Wallace, 1974). These ambiguous results could be likely explained by the variation in experimental methodologies and conditions, which were found to have profound effects on the outcome of the studies (McCusker, Bryant and Gardiner, 2015). Moreover, Schwann cells (Wallace and Wallace, 1973) and skeletal muscle (Thornton, 1938a; Hay, 1959; Hay and Fischman, 1961; Cameron, Hilgers and Hinterberger, 1986) were also found to be important cell sources for blastema formation. In particular, skeletal muscle has been extensively studied in this process (see section 2.4.2) and became over time the classical system to address the question of blastemal origin in limb regeneration (Brockes, 1997; Simon and Tanaka, 2013). A more recent study, using tissue-specific GFP-labelling, tracked the major limb tissues and confirmed that dermal fibroblasts, Schwann, skeletal and myogenic cells all contributed to blastema formation (Kragl *et al.*, 2009). Importantly, the same study also found that, despite its homogeneous morphology, blastemal cells retained memory of their tissue origin, demonstrating their restricted potential in re-differentiating into different tissues. The origin of the limb blastema is far from being solved, but the development of new tools that allow for the tracing of specific tissues, as the one previously mentioned, will likely be instrumental to explore the issue.

Θ Nerve dependence

Since limb regeneration was discovered, the study of this intricate system has produced a rich body of literature, providing us with a better understanding of its intrinsic properties. One of the most studied features of limb regeneration is the role of the nerves (Stocum,

2011). Nerve dependence was first discovered by Tweedy Todd in 1823, who showed that limb denervation could either impair or completely abolish regeneration, depending on the stage it was performed (Todd, 1823; Dinsmore, 1991). Nonetheless, these findings had no significant impact on the scientific community and went relatively unnoticed until the next century. Here, studies investigating the role of nerves in embryonic development eventually revived the interest in exploring it also in the context of regeneration. Early experiments by scientists such as Goldfarb, Locatelli and Schotté were fundamental in the field to fuel the debate and generated substantial disagreement on this issue. While some denied the nerve influence in regeneration, others argued that successful regeneration upon denervation was the result of inadequate procedures, or poor maintenance of the denervated state (Singer, 1952; Wallace, 1981).

Several studies followed where the nerve control of early regeneration was thoroughly investigated and, ultimately, it became generally accepted that regeneration was a nerve-dependent process (Fig. 7). First, it was observed that limb denervation, typically performed through nerve transection, leads to neuronal disintegration (chromatolysis) (Tweedle, 1971) and degeneration of the distal part of the axons, a process known as Wallerian degeneration (Singer, 1946), named after its discoverer (Waller, 1850). Later on, Schotté's detailed investigation through denervation experiments was instrumental to establish that limb regeneration was highly dependent on the presence of nerves (Butler and Schotté, 1941; Schotte and Butler, 1941; Schotté and Butler, 1944). This was followed by a long series of studies performed by Marcus Singer, where he made several crucial findings: 1) Both motor and sensory nerves contribute for regeneration; 2) The critical factor for regeneration to occur is not the type, but the extent of innervation. Regeneration occurs if the number of axons in the amputation area is above a certain threshold; 3) The threshold varies according to the position along the proximal-distal (PD) axis of the limb (Singer, 1952). Based on this work, Singer developed the neurotrophic hypothesis, which stated that the survival and proliferation of the blastema required certain chemical factors that were provided by the nerves (Stocum, 2011). This theory was essential to set the foundations for the future studies, which focused on identifying neurotrophic factors that would mediate this regulatory function on regeneration.

The most notable exception to this theory emerged when studies with aneurogenic limbs (i.e. nerve deprived) reported their capacity to regenerate (Fig. 7) (Yntema, 1959, 1959). This can be achieved by excising the neural tube of an embryo, which still develops relatively normally, but lacks the capacity to move or eat. Consequently, their survival can only be ensured if joined in parabiosis with a normal larva and sharing a common circulation. These experiments were fundamental to determine that while nerve dependence originates during limb development, regeneration does not become nerve-dependent if limb innervation is prevented (Stocum, 2011). This provides a useful

distinction between development and regeneration. The phenomenon of the aneurogenic limb has led many to question the validity of the neurotrophic theory, but instead of abandoning it completely, some modifications were proposed so the theory could also accommodate these cases. In the modified version of the trophic theory it was considered that other tissues can also produce their trophic substance initially but, upon innervation, they become dependent of the presence of nerves and the neurotrophic factor (Wallace, 1981). Thus, the modified version still maintained the idea that limb regeneration was quantitatively dependent on some factor that was delivered by the axons. So the pursuit for such a factor continued.

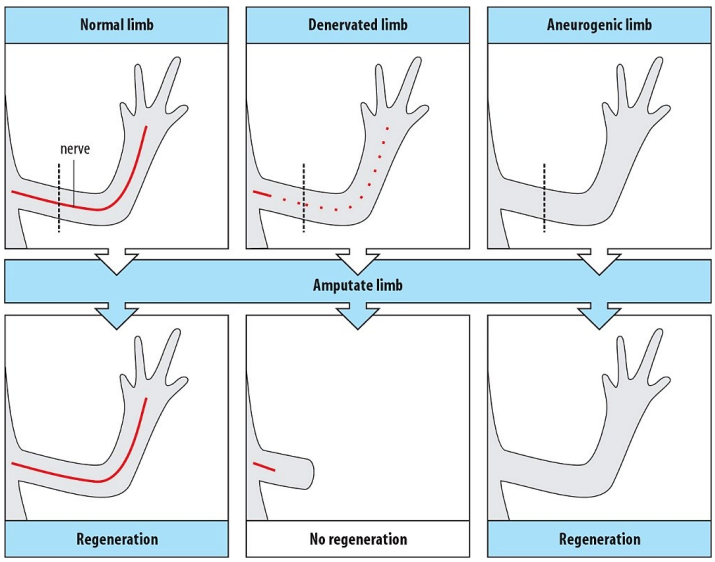

Figure 7: Nerve dependence of salamander limb regeneration. Denervated limbs, where the nerve is transected, fail to regenerate, whereas nerve-deprived limbs (aneurogenic) can complete regeneration. (From: http://www.mun.ca/biology/desmid/brian/BIOL3530/DEVO_13/devo_13.html)

Currently, we are still lacking a complete understanding of the nerve roles in regeneration, but some relevant factors, which support the trophic theory, have been identified so far. Over 30 years ago, Glial growth factor 2/Neuregulin-1 (Ggf2/NRG1) was proposed as neurotrophic factor as it was shown to be present in the blastema, expressed by the dorsal root ganglia (DRG) neurons and its levels become reduced upon denervation (Brockes, 1984). Additionally, more recent studies have demonstrated that Ggf2 has the potential to rescue regeneration in denervated limbs, and that is expressed in the basal cells of the wound epidermis and most of the blastema (Wang, Marchionni and Tassava, 2000; Farkas *et al.*, 2016). Another interesting case is the newt anterior gradient (nAG) protein. This secreted protein has also been shown to be sufficient to rescue regeneration

in denervated limbs (Kumar et al., 2007). Furthermore, this component was observed to act as a ligand for a blastema cell-surface protein called Prod1, which is expressed in a gradient along the proximal-distal axis of the limb (Da Silva, Gates and Brockes, 2002). This protein receptor was identified as an important component that determines a proper positional identity in regenerating tissues (Kumar, Gates and Brockes, 2007) and helped uncovering an important regulatory mechanism of regeneration.

Recently, different combinations of other factors such as FGF and BMP proteins have also emerged as interesting neurotrophic candidates, as they are sufficient to replace the absent nerve in certain conditions (Makanae, Mitogawa and Satoh, 2014; Satoh *et al.*, 2016). Interestingly, these studies were conducted using the accessory limb model (ALM), an alternative system designed to study limb regeneration without amputation (Endo, Bryant and Gardiner, 2004). Instead, it involves the removal of a piece of skin and the deviation of a nerve to the wound site. This induces the formation of a blastema and triggers the regeneration of an ectopic limb, providing a useful model to investigate the roles of new nerve factor candidates. Altogether, these results strongly support Singer's neurotrophic theory and point towards an interdependent relationship between the nerves, the wound epidermis and the blastema cells.

Θ The immune system

Regeneration studies have also focused in other important properties such as the roles of the immune system. An interesting perspective is that differences in the immune system operating mechanisms, might account for differences in regenerative capacity. This has been proposed upon the observation that regeneration inversely correlates with the maturation of the immune system (Godwin and Brockes, 2006). Frogs, which are evolutionarily close to salamanders, are a good example to illustrate this point, since they gradually lose regenerative potential and scar-free repair during development, while the immune system matures (Bertolotti, Malagoli and Franchini, 2013).

The notion that the immune system impinges on salamander limb regeneration is not new. In fact, it has been previously addressed in several studies, where irradiation or immunosuppressant therapies have been shown to affect regeneration (Mescher and Neff, 2006; Godwin and Rosenthal, 2014). However, with the development of new molecular tools and the consequent improvement in our understanding of the immune system features and associated roles, this issue is now being revisited. This renewed interest led to the discovery that macrophages are essential to mediate the early response to injury and, when depleted systemically, limb regeneration fails due to extensive fibrosis and dysregulation of extracellular matrix (ECM) components (Godwin,

Pinto and Rosenthal, 2013). In other words, some components of the immune response help creating a permissive environment for regeneration. Moreover, a follow up study found macrophages to be also necessary for salamander heart regeneration and the identified mechanisms recapitulate those observed in the limb (Godwin *et al.*, 2017). Remarkably, similar observations have been made in zebrafish where macrophages were observed to modulate tail fin regeneration (Petrie *et al.*, 2015), suggesting that different species might rely on the same general mechanisms to ensure regeneration.

In mammals, the well documented roles of the immune system in inflammation and scar formation are best illustrated by the fact that embryos are capable of scar-free wound healing until they start developing certain immune cell types (Mescher and Neff, 2005). Recently, work performed with the African spiny mouse elucidated a similar requirement for macrophages to elicit a regenerative response upon injury (Simkin *et al.*, 2017). This further establishes macrophages (and inflammation) as interesting targets for regeneration studies and might have a positive implication in our aspirations of improving mammalian regeneration.

2.4.2 Skeletal muscle dedifferentiation

Skeletal muscle in vertebrates is mainly composed by long multinucleated fibers (myofibers), which are surrounded by a network of blood vessels and nerves, and bound together by layers of ECM. They are formed during development, in a process called myogenesis, where precursor mononucleated cells (myoblasts), commit to differentiation by exiting the cell cycle (myocytes) and fuse to form multinucleated myotubes (Fig. 8), which will later mature into myofibers (Fig. 9) (Stockdale and Holtzer, 1961; Buckingham, 2001; Dumont *et al.*, 2015; Hernandez-Torres *et al.*, 2017). Although skeletal muscle has previously been described as an important source of the blastema, the mechanisms by which cells are derived from this tissue have been a subject of long and controversial discussions (Brockes, 1997; Slack, 2006). In this context, dedifferentiation refers to fragmentation of the myofibers during appendage regeneration, in a process called cellularization, with the muscle-derived mononucleate progeny contributing to the blastema, where they proliferate.

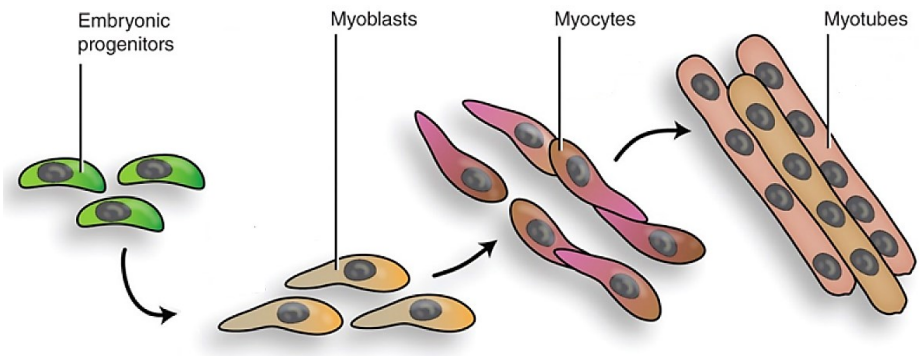

Figure 8: Skeletal myogenesis occurs from precursor cells that are mononucleated and can proliferate. After several rounds of proliferation, myoblasts exit the cell cycle and become myocytes. Myocytes can undergo a fusion process to form multinucleated myotubes that eventually mature into myofibers. (Reproduced with permission and adapted from: Dumont *et al*., 2015).

Several studies have attempted to show regeneration happening via dedifferentiation, first based on histological observations (Chalkley, 1954; Hay, 1959) and later through short-term labelling of myofibers or transplanted cultured myotubes (Namenwirth, 1974; Lo, Allen and Brockes, 1993; Kumar et al., 2000; Echeverri, Clarke and Tanaka, 2001). During the same period, it was discovered that myofibers in frog and rat muscle harbor a population of resident stem cells (Katz, 1961; Mauro, 1961), named satellite cells for their location between the plasma membrane of the myofiber (sarcolemma) and the basal lamina (Fig. 9). These cells were later found to be a common feature among vertebrates,

including salamanders (Popiela, 1976), thus challenging the idea of dedifferentiation and sparking the debate. For many years, electron microscopy was the only definitive method of identification of satellite cells but, more recently, several molecular markers have been described that can be used to label these cells (Dumont *et al.*, 2015). One of the most prominent of those markers is Paired box protein 7 (Pax7), a transcription factor which allowed for the identification and characterization of a population of satellite cells that becomes active in newts upon limb amputation (Cameron, Hilgers and Hinterberger, 1986; Morrison et al., 2006; Kragl et al., 2009). Moreover, this population was observed to be stable and maintained in the regenerated limb after repeated amputations (Morrison, Borg and Simon, 2010). Nevertheless, due to the limitations of the available techniques, a conclusive answer based on quantitative estimations and long-term labelling that would allow to fate-map endogenous myofibers, was still lacking.

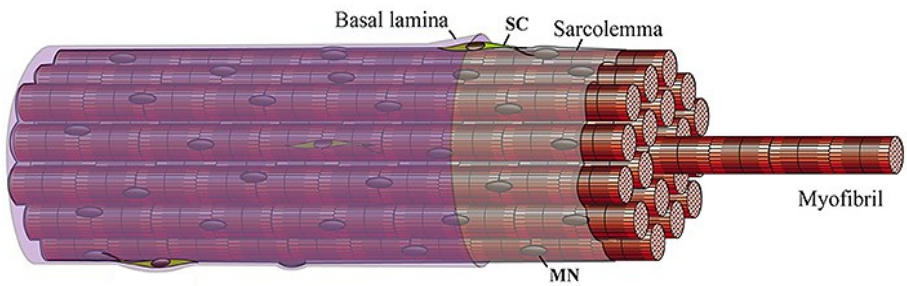

Figure 9: Subcellular architecture of skeletal myofibers. Mature myofibers containing a high number of myonuclei (MN) and are formed by a bundle of myofibrils, which provide the contractile properties. In addition, satellite cells (SC), a population of resident stem cells is localized between the myofiber membrane (sarcolemma) and the extracellular matrix layer (basal lamina), and plays important roles in muscle repair and regeneration. (Adapted from: Hernandez-Torres *et al.*, 2017)

More recently, this situation has changed with the emergence of new techniques which contributed to overcome this gap. Namely, through long-term tracing of muscle-derived progenitor cells, it was demonstrated that skeletal muscle dedifferentiation is an integral part of limb regeneration in newts (Sandoval-Guzmán et al., 2014). In contrast, the same study found no evidence of muscle dedifferentiation in the limb of another salamander species, the axolotl. Instead, we observed that muscle regeneration in this species is exclusively mediated by the activation of $Pax7^{+}$ satellite cells in the outgrowing limb (Fig. 10). These results show how closely related species might display different strategies to regenerate limbs after injury. Interestingly, mammals are also capable to regenerate injured skeletal muscle, stimulated by the activation of satellite cells, even though this regeneration does not involve the formation of a blastema and depends on the type and

severity of the injury (Chargé and Rudnicki, 2004; Turner and Badylak, 2012). Currently, the remaining challenge is to understand the what mechanisms trigger skeletal muscle dedifferentiation.

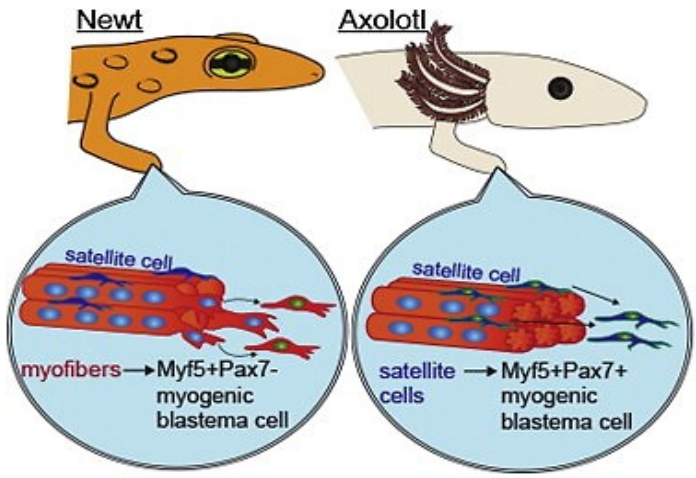

Figure 10: Limb regeneration mechanisms between different salamander species. In the newt, myofiber fragmentation results in proliferating, $Pax7^-$ mononuclear cells in the blastema that give rise to the skeletal muscle in the new limb. In contrast, myofibers in axolotl do not generate proliferating cells, and do not contribute to newly regenerated muscle. Instead, resident $Pax7^+$ cells provide the regeneration activity. (Reproduced with permission from: Sandoval-Guzmán *et al*. 2014).

Θ Initiation of dedifferentiation

To understand how a terminally differentiated cell type in a stable quiescent state reverts its differentiated profile, a number of groups have attempted to uncover how dedifferentiation is initiated. Another key question is to what extent such mechanisms are conserved and inducible in mammalian cells. In this context, a study using mammalian cultured myotubes found that compounds, which induce microtubule depolymerization, such as myoseverin, had the potential to induce fragmentation of the multinucleated muscle cell (Rosania *et al.*, 2000). However, a more thorough analysis, through time lapse microscopy, determined that the generated mononucleate progeny did not survive and, therefore, failed to resume the cell cycle (Duckmanton *et al.*, 2005). Although other studies presented evidence for myotube fragmentation leading to proliferating mononucleate cells, it is worth mentioning that they did not use proper lineage-tracing tools, so it should not be excluded that these cells could have originated from pre-existent mononucleate cells in the cultures (Odelberg, Kollhoff and Keating, 2000; McGann, Odelberg and Keating, 2001; Kumar *et al.*, 2004; Jung and Williams, 2011).

In the last few years, substantial progress has been made in the development of new fate-mapping techniques. Taking advantage of these tools, one recent study established a direct association between programmed cell death (PCD) and myogenic dedifferentiation (Wang *et al.*, 2015). Through *in vivo* molecular manipulations, they observed that newt muscle dedifferentiation depended on an apoptotic response that was not fully executed (Fig.11). Furthermore, by studying cultured myotubes, they demonstrated that it was possible to derive proliferating progeny from terminally differentiated muscle cells by first initiating a PCD response and subsequently blocking the full execution of the apoptotic process. In contrast, mammalian myotube-derived mononucleate cells only resumed proliferation upon knockdown of p53 gene, thus providing a key platform to address differences between newts and mammals. Overall, these evidence might reflect an evolved strategy in newts to divert an injury-mediated cell death response into mechanisms that fuel regeneration.

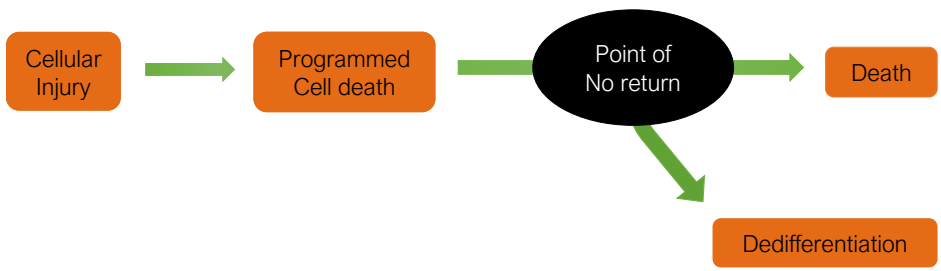

Figure 11: Model for initiation of dedifferentiation during newt limb regeneration. Cellular injury typically initiates a programmed cell death response which ultimately results in cell death. However, if the full execution of the programmed cell death process is prevented at an adequate point, cell survival can be manifested in the production of dedifferentiated cells. This suggests that cell dedifferentiation might be induced through an apoptotic response that is not fully executed. (Based on: Wang et al. 2015)

Θ Cell cycle re-entry

Tissue regeneration can occur through distinct general mechanisms such as dedifferentiation and/or transdifferentiation (Jopling, Boue and Belmonte, 2011). Dedifferentiation, as already described, involves terminally differentiated cells that regress to a less-differentiated stage within the same cell lineage and then proliferate to replace the missing tissue. Aside from the case of the newt muscle, this event can also be observed in cardiomyocytes during zebrafish heart regeneration (Jopling *et al.*, 2010; Kikuchi *et al.*, 2010), and in mammalian myelinating Schwann cells upon nerve damage

(Chen, Yu and Strickland, 2007). Transdifferentiation allows cells to dedifferentiate even further, to a point where they can switch lineage and re-differentiate into a different cell type. This phenomenon takes place during newt lens regeneration and it was first observed by Gustav Wolff in 1894 (Reyer, 1954). Surgical removal of the lens (lentectomy), induces the pigment epithelial cells (PECs) from the dorsal iris to dedifferentiate and re-enter the cell cycle to create a new lens vesicle that generates the new cells of the lens (Tsonis, Madhavan, Tancous, *et al.*, 2004). Importantly, this occurs without contribution of the ventral PECs, as only the dorsal PECs transdifferentiate *in vivo* to form the new lens. Another relevant process to take into account is cellular reprogramming, where differentiated cells revert to a pluripotent state, which can give rise to almost any cell type (Jopling, Boue and Belmonte, 2011). However, reprogramming into pluripotency has not been established as an actual regenerative response and is mostly induced artificially for potential therapeutic purposes approaches (Yamanaka and Blau, 2010). Whereas cell cycle re-entry has been shown to be non-essential for dedifferentiation or transdifferentiation to happen (Tsonis, Madhavan, Call, *et al.*, 2004; Monje *et al.*, 2010), it is still necessary for proper regeneration. This emphasizes the importance of identifying molecular pathways that induce the proliferation of dedifferentiating muscle cells.

In the late nineties, a study reported that cultured newt skeletal myotubes re-entered the cell cycle when exposed to serum, which occurred through phosphorylation of the retinoblastoma (Rb) protein (Tanaka *et al.*, 1997). This contrasted with previous observations in mammalian myotubes (Olwin and Hauschka, 1988) and pointed towards the presence of certain components in serum that could induce myotubes to revert their post-mitotic state. A follow up study from the same lab, showed that treatment with serum proteases which regulated blood clotting, such as Thrombin and Plasmin, strongly enhanced the effect of cell cycle re-entry (Tanaka, Drechsel and Brockes, 1999). In addition, Thrombin proteolytic activity was found to be elevated in the end of the stump from regenerating limbs. The underlying mechanism of this phenomenon remained elusive, but it was proposed that Thrombin was an indirect mediator generating some factor that acted on the myotubes. Overall, these studies contributed to establish an important link between injury response and dedifferentiation. This association gained further support when similar effects of Thrombin were observed in newt lens regeneration (Simon and Brockes, 2002) and by the fact that an extract from newt regenerating limbs had the potential to induce dedifferentiation of mammalian myotubes (McGann, Odelberg and Keating, 2001). These compelling results greatly encouraged scientists to pursue the factors that were cleaved by clotting proteases and triggered cell cycle re-entry during regeneration.

Recent findings have identified a MARCKS-like protein (MLP), which was expressed in epithelial cells and promoted the proliferation of both resident stem cells and myofiber-derived progeny (Sugiura *et al.*, 2016). Although this factor stands out as an important component for triggering dedifferentiation, its connection to serum proteases remains unclear. Several canonical signaling pathways have also been implicated in amphibian regeneration, such as WNT- , FGF- (Hayashi, Mizuno and Kondoh, 2008; Lin and Slack, 2008), and BMP-signaling (Grogg *et al.*, 2005; Beck *et al.*, 2006), typically on the basis that inhibition of some components impaired the regenerative response. These results raised the possibility that these pathways might be activated by injury, a hypothesis that has been explored in **Paper III**, where we identified components from the BMP pathway which acted as downstream targets of serum proteases and induced cell cycle re-entry of myotubes.

2.5 Regenerative medicine and future directions

One of the main reasons why the field of regeneration research appeals to most people, is the prospect of inducing regeneration of structures that do not have that capacity. The rich history of regeneration research has generally contributed to raise the hopes of translating the acquired knowledge into clinical applications. Regenerative medicine, as it has been labeled, aspires to repair or replace tissue or organ functions which have been lost due to age, disease, injury or developmental defects. This field aims to do so, either by stimulating damaged tissues/organs to self-repair or, when not possible, to grow them in the lab for transplantation (Maienschein, 2011; Terzic *et al.*, 2015). Stimulating human limb regeneration has proved to be an elusive goal so far, but considerable progress has been made both in cell therapy and tissue engineering.

Cell-based therapies include a variety of cell sources for tissue repair such as stem, progenitor, tissue-specific primary cells and stem cell derivatives. Depending on the therapeutic approach, the cells can be injected intravenously, transplanted into the injury site or recruited from the patient's tissues to stimulate self-repair (Buzhor *et al.*, 2014). Stem cells are regarded as the most suitable option due to their potential for self-renewal and differentiation. Furthermore, tissue-specific resident stem cells have the capacity to migrate to the affected area, differentiate and replace the damaged cells (Blau, Brazelton and Weimann, 2001). Nevertheless, since this self-repair mechanism is often insufficient to revert the pathological processes in many diseases, external cell therapy is usually necessary. In these cases, the cells used can be either derived from the same patient (autologous) or derived from a donor (allogeneic). The former has the advantage of posing a low risk of adverse immune reaction, whereas the latter allows for off-the-shelf tissues to be produced in big scale (Mao and Mooney, 2015; Mount *et al.*, 2015).

The majority of cell-based therapies is currently still experimental or undergoing clinical trials (Ankrum and Karp, 2010; Buzhor *et al.*, 2014), but there are some exceptions that successfully made it into the clinic. Since regenerative medicine emerged as an industry, a dozen of therapies have received approval from the regulating agencies and reached the market (Mao and Mooney, 2015). One of the best cases is the well-established haematopoietic stem cell transplantation, which is widely used in the treatment of blood related disorders (Weissman and Shizuru, 2008). Other examples of the already approved therapies include autologous chondrocytes for the treatment of articular cartilage defects (Dewan *et al.*, 2014), autologous keratinocytes to repair severely burnt skin (Gardien, Middelkoop and Ulrich, 2014) and allogeneic fibroblasts to treat diabetic foot ulcers (Harding, Sumner and Cardinal, 2013).

Tissue engineering, which can be considered a sub-field within regenerative medicine has also made substantial progress (Fig. 12) (Khademhosseini and Langer, 2016). This is linked to the steady development of new biomaterials, since tissue engineering typically depends on scaffolds that provide the proper architecture, onto which cells are seeded to develop into the new organ, in an organized fashion (Mao and Mooney, 2015). Notably, the development of tissue-engineered skin and bladders has advanced rapidly, fueled by emerging techniques such as 3D-Bioprinting (Atala *et al.*, 2006; Tarassoli *et al.*, 2018). A critical component of the biomaterials used in these products is how they integrate into the host environment, thus making biocompatibility and biodegradability essential features. Ultimately, the goal is to develop materials that do not lead to adverse responses or toxic byproducts, and that degrade at a similar rate to the growth of new tissue, at the

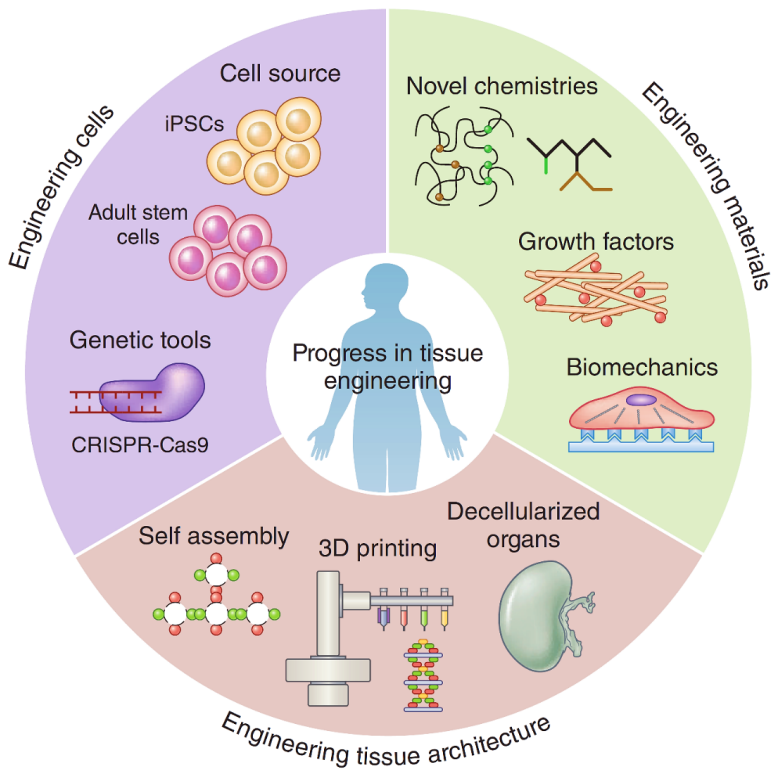

Figure 12: Summary of tissue engineering progress in the past decade. Additional cell sources have become available, including induced Pluripotent Stem Cells (iPSCs) and adult stem cells, as well as genetic editing tools that enable greater cell manipulation. Improved chemistries and growth factor delivery mechanisms, as well as advances in understanding biophysical cues on cellular behaviors and tissue architecture technologies have contributed to engineering tissues of considerably improved structural, compositional and functional resemblance to their native counterparts. (Reproduced with permission from: Khademhosseini and Langer, 2016).

site where they are implanted (Lee, Kasper and Mikos, 2014). Because function is strongly dependent on tissue and organ architecture, the ability of scaffolds to recreate structure, usually determines the success in recapitulating the healthy tissue (Nelson and Bissell, 2006). One way to faithfully capture organ architecture in engineered tissues is to decellularize organs and then seed them with cells before transplantation. This allows for removal of cells and molecules that could trigger an immunogenic response, while maintaining the organ structure and its native mechanical properties and extracellular matrix (Crapo, Gilbert and Badylak, 2011).

Overall, the efficacy of regenerative medicine based therapies has been variable, but some products were shown to perform better or are at least comparable to previous treatments (Dewan *et al.*, 2014). Moreover, several cell therapy applications are in advanced developmental stages and hold great translational promise for the treatment of several prominent disorders (Buzhor *et al.*, 2014). However, it is important to bear in mind that it is still a considerable challenge to bring such therapies to the market. First, because earning market approval for these products typically entails long time investments and high costs (Mount *et al.*, 2015). Second, because cell-based products, which are to be transplanted to patients, harbor several potential risks such as the potential to form tumors (tumorigenicity) or the possibility of immune rejection (immunogenicity) (Heslop *et al.*, 2015). This requires a balance to be established between minimizing potential risk and ensuring that new treatments are not kept from patients unnecessarily. Naturally, this depends on an accurate assessment of the associated risks but, as therapy safety is still poorly understood, it is vital to be cautious in the translation of potential therapies to the clinic. Overall, despite the important advances that have been made, all these factors should be considered to avoid unrealistic expectations towards the future of regenerative medicine.

3. The pursuit of the salamander genome

Salamanders form one of the main groups of amphibians. They are typically grouped into ten different families, which display considerable biological differences between and within them (Brockes, 2015). For instance, while many salamander species undergo a process of metamorphosis as they develop into adults, others are paedomorphic, which means they retain their larval features during adulthood (Johnson and Voss, 2013). Moreover, many species from the family *Plethodontidae* (or lungless salamanders), have a direct development from embryo to adult, without going through larval stages, as it is typical of most species (Gómez *et al.*, 2017). Interestingly, as previously mentioned, the mechanisms which are employed for limb regeneration by different species can also vary (Sandoval-Guzmán *et al.*, 2014). This highlights the importance of taking interspecific variations into account, particularly when attempting to understand the processes behind the regenerative capacities of salamanders. To do so, it is fundamental to be able to investigate genome regulation and its evolution, however, the lack of genomic resources has been a long-lasting limitation in the field. In this chapter, I will discuss how the evolution of the sequencing technologies allowed the sequencing and assembly of the first salamander genomes and the main obstacles that have prevented a faster progress in the area until very recently.

3.1 The giant genomes of salamanders

Among vertebrate species, most of the largest genomes are found in salamanders, ranging from ~14 – 120 Gigabases (Gb) (Fig. 13) (Brockes, 2015). For comparison, this is much larger than the human genome, which is about 3.5 Gb (Gregory, 2018). As in other organisms, genome size in salamanders positively correlates with chromosome size, but not with chromosome number (Olmo and Morescalchi, 1975; Gregory, 2005; Sessions, 2008). Consequently, harboring a larger genome is usually linked with an increase in cell size and cell cycle length, which has an impact on several biological aspects of an organism. Salamander species with larger genomes typically display slower rates of metabolism, embryonic development and regeneration than species with smaller genomes (Gregory, 2003; Litvinchuk, Rosanov and Borkin, 2007; Sessions, 2008).

So what are the causes behind such gigantic genomes? While this is not yet fully understood, several studies have showed that highly abundant transposable elements (TE) and long intronic sequences were two important features in salamanders (Batistoni *et al.*, 1995; Marracci *et al.*, 1996; Smith *et al.*, 2009; Zhu *et al.*, 2012). TEs, in particular,

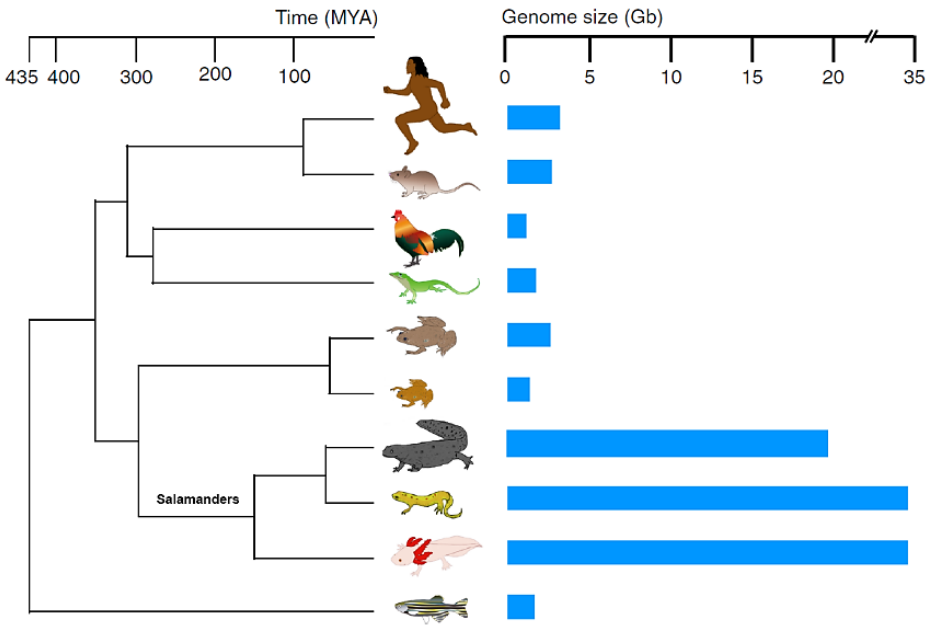

Figure 13: Salamanders have the largest genomes when compared to other vertebrate groups. The species represented are (from the top): Human (*Homo sapiens)*, Mouse (*Mus musculus)*, Chicken (*Gallus gallus*), Lizard (*Anolis carolinensis*), Frogs (Xenopus laevis, Xenopus tropicalis), Salamanders (*Pleurodeles waltl*, *Notophthalmus viridescens*, *Ambystoma mexicanum*) and Zebrafish (*Danio rerio*). MYA-Million years ago, Gb-Gigabases (Adapted from: Elewa *et al*. 2017).

which are mainly divided between retrotransposons (class I) and DNA transposons (class II) (Wicker *et al.*, 2007), have been shown to cover up to 47% of the entire genome in some species (Sun *et al.*, 2012). In addition, this same study found the long terminal repeat (LTR) retrotransposon to be a highly represented category and account for roughly one-third of the genome. The existence of these overrepresented elements offers relevant candidates to be investigated in the context of regeneration. Consistent with this hypothesis, another family of retrotransposons, the non-LTR long interspersed nucleotide element- 1 (LINE-1), was recently implicated in axolotl limb regeneration (Zhu *et al.*, 2012). This study found that LINE-1, which is typically active in germ cells, was highly upregulated during limb regeneration, establishing a germ-like state that might play a role in stimulating cell dedifferentiation.

Although salamanders display remarkable features as a regeneration model, the absence of a reference genome has negatively impacted the development of new genetic tools, which could contribute to new knowledge regarding the molecular pathways involved in regeneration. For a long time, this has remained an elusive goal as the large genome size, the abundance of repetitive elements and the lack of a closely related genome, made it a time-consuming, costly, and challenging task (Looso *et al.*, 2013; Looso, 2014).

3.2 Genome sequencing

To understand the limitations associated with sequencing such large genomes, it is important to place it into the context of the history of sequencing technologies. Since it was developed in the 1970s, the method of Sanger sequencing (Sanger, Nicklen and Coulson, 1977) has remained as the gold standard for at least 30 years. This technique transformed biology as it supplied scientists with the necessary tools to analyze entire genes and, later, full genomes. However, the growing demand for higher throughput fostered the development and commercialization of next-generation sequencing (NGS) technologies. The first of its kind was released in 2005 and it received this name to highlight the improvement in comparison with Sanger sequencing, which was defined as a first-generation technology. The higher scalability provided by NGS, fueled the sequencing of entire genomes at an extraordinary speed (Shendure and Ji, 2008; van Dijk *et al.*, 2014).

Whereas this NGS revolution allowed for the sequencing of many species genomes, it had a considerable disadvantage as these technologies generated relatively short reads. This turned genome assembly into a more complex task and relied on the development of new alignment algorithms (van Dijk *et al.*, 2014). Naturally, this was particularly problematic for large genomes such as the ones from salamanders. Nonetheless, these technologies still had an important role in salamander research, as they promoted several efforts to generate transcriptomic and proteomic resources from different species including newts (Abdullayev *et al.*, 2013; Looso *et al.*, 2013), axolotls (Stewart *et al.*, 2013; Wu *et al.*, 2013; Bryant *et al.*, 2017; Caballero-Pérez *et al.*, 2018) and others (Che *et al.*, 2014; Nakamura *et al.*, 2014). Although they have led to valuable new insights and working tools for the study of regeneration, these resources are limited and do not allow large-scale analysis of species-specific genes or gene family evolution.

Later on, new methods have emerged to handle the NGS shortcomings, the so called third-generation sequencing, which allowed for much longer reads than ever before, making it suitable for *de novo* genome assemblies (van Dijk *et al.*, 2014). These advances in sequencing technologies became a decisive factor to overcome previous constraints. Ultimately, this resulted in the recent publication of two studies where the genomes of the Iberian-Ribbed newt (*Pleurodeles waltl*) (**Paper I**) and the Mexican axolotl (*Ambystoma mexicanum*) (Nowoshilow *et al.*, 2018) were successfully sequenced and assembled. This is a major leap forward which opens up new possibilities and will be a driving force to generate new momentum in the field.

3.3 Genome editing and transgenic lines

Despite being an attractive model to study regeneration, salamanders have features that make it a challenging animal model to work with. Apart from the large genome size, some salamander species, as in the case for newts, also reproduce through a complex and long life cycle (Fig. 14), making it more difficult to breed under laboratory conditions, when compared to other model organisms. These factors have greatly restricted genetic studies and the efforts in establishing salamander transgenic lines (Kumar and Simon, 2015). Gene function studies have traditionally relied on tools like ectopic overexpression or transient knockdown via morpholinos of genes of interest (Kumar *et al.*, 2004; Schnapp and Tanaka, 2005). However, alternative techniques which can remove or completely inactivate genes are desirable as they can yield more definitive evidence when evaluating the implication of those genes in regeneration (Housden *et al.*, 2016).

In spite of the numerous challenges, some labs have succeeded in establishing transgenic salamander lines. While transgenic animals have been generated for different newt species (Casco-Robles *et al.*, 2011; Hayashi *et al.*, 2013), most of the recent developments were achieved in axolotl (Khattak *et al.*, 2013). These lines led to valuable findings such as: 1) The different cells in the blastema retain the information of their tissue origin during limb regeneration (Kragl *et al.*, 2009); 2) Blood stem cells do not contribute

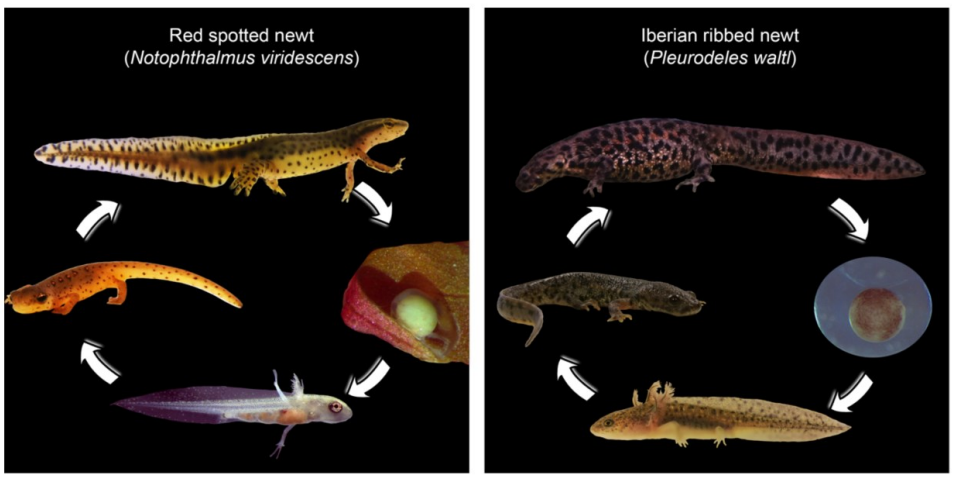

Figure 14: Life cycle of two newt species commonly used in research. Both of these species (Red-spotted and Iberian-ribbed newts) display a similar cycle that typically includes: Embryonic development from the egg stage (Right) into the fully aquatic larva (Bottom). The larvae subsequently undergo metamorphosis into juveniles/efts (Left), which will then grow and sexually mature as they turn into adults (Top). (Figure by: Alberto Joven)

to muscle or nerve formation during tail regeneration, showing no significant plasticity (Sobkow *et al.*, 2006); 3) The induced expression of the mammalian tumor suppressor $p16^{INK4a}$ negatively impairs spinal cord regeneration (Khattak *et al.*, 2013). Recently, the rapid development of CRISPR-Cas9, a breakthrough technology which enables genome engineering in a specific and simple way, has completely transformed biological research (Doudna and Charpentier, 2014). In particular, this became a crucial resource to generate new salamander genome edited lines, which helped uncovering new functional features of factors such as Sox2, Pax3 and Pax7 during tail (Fei *et al.*, 2014) and limb regeneration (**Paper I**; Fei *et al.*, 2017; Nowoshilow *et al.*, 2018). Together with the recently sequenced salamander genomes, this technology will likely be shaping the future of salamander research and regenerative biology in years to come.

4. Non-coding small RNAs

During the past two decades, we have seen an exponential increase of identified RNA transcripts that are not translated into proteins. These have been generally classified as non-coding RNAs (ncRNAs), a new class that strikingly contrasts to the traditional functions of messenger RNA (mRNA) (Fig. 15) (Santosh, Varshney and Yadava, 2015). While a few examples of ncRNAs were already known previously, such as ribosomal RNA (rRNA) and transfer RNA (tRNA), they have never been considered separately, due to their roles in protein-translation machinery (Hüttenhofer, Schattner and Polacek, 2005). Since Molecular Biology was, until this point, focused on protein-mediated regulation, where non-coding genes were thought to be "junk DNA", the emergence of ncRNAs has contributed to a paradigm shift in the field, with some labelling it as the Noncoding RNA revolution (Cech and Steitz, 2014) for its profound impact on post transcriptional regulation of gene expression. In fact, it has been estimated that in the entire human genome, only ~1% of genes actually encode for proteins (Rands *et al.*, 2014), meaning that, until recently, most of our genome remained largely unexplored.

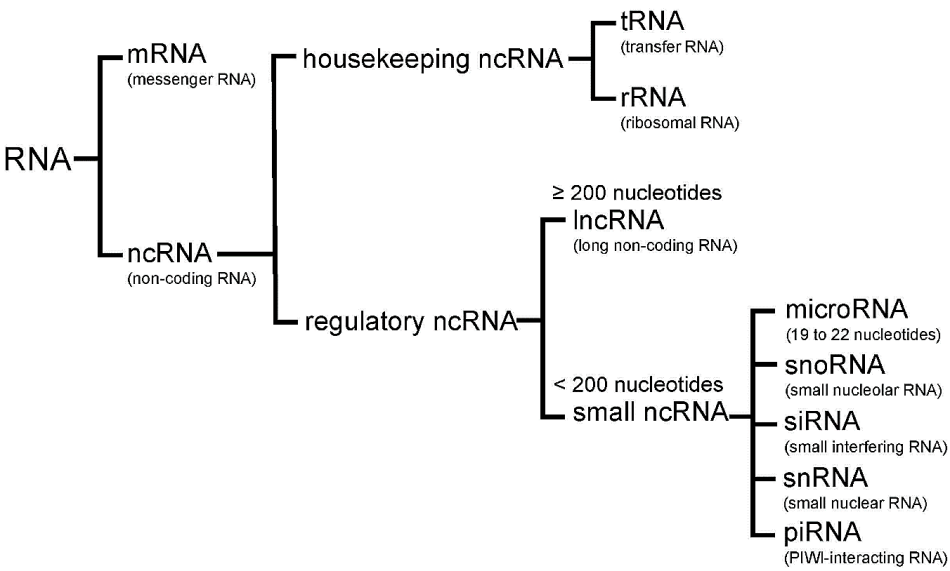

Figure 15: Diversity of RNA categories. RNAs are divided into two major classes: coding, which corresponds to messenger RNA (mRNA), and non-coding RNA (ncRNA). NcRNAs are divided between housekeeping ncRNAs, which are involved in protein translation and consist of transfer RNA (tRNA) and ribosomal RNA (rRNA), and regulatory ncRNAs. Regulatory ncRNAs are classified based on their size into long ncRNA (lncRNA) and small ncRNA. Small ncRNAs are subclassified into microRNA (miRNA), small nucleolar RNA (snoRNA), small interfering RNA (siRNA), small nuclear RNA (snRNA), and PIWI-interacting RNA (piRNA). (From: Inamura, 2017)

Among other factors, one of the main driving forces that was fundamental to bring the ncRNAs into the spotlight was the development of the NGS technologies, which achieved a level of resolution that was not possible before (Metzker, 2010). Consequently, this led to a substantial increase in the number of large scale sequencing studies in a variety of organisms and tissues, which have greatly contributed to characterize all the ncRNAs we know today. Currently, many classes of ncRNAs have been identified so far and they are primarily distinguished based on their sizes, between short non-coding RNA (sncRNAs) with < 30 nucleotides (nts) and long non-coding RNA (lncRNAs) with > 200 nts (Santosh, Varshney and Yadava, 2015; Inamura, 2017).

LncRNAs consist in a very heterogeneous class of RNAs that includes thousands of different species with a big range of sizes. They can originate from various locations in the genome from introns to intergenic regions and have been shown to regulate gene expression at different levels. LncRNAs can function both as ligands for proteins and as mediators that guide regulator complexes to specific DNA or RNA target sites. This flexible scaffold nature is critical to link proteins and/or other RNAs that would not interact otherwise (Fatica and Bozzoni, 2014).

The short non-coding category includes several established groups where microRNAs (miRNAs) represents the most prominent and well-studied (described in detail in the next section). Other classes include small interfering RNAs (siRNAs) that can act through different pathways to regulate gene expression at transcriptional and post-transcritptional level (Claycomb, 2014), small nuclear (snRNAs) and small nucleolar RNA (snoRNAs), which are important components of the splicing machinery (Matera, Terns and Terns, 2007) and PIWI-interacting RNAs (piRNAs), best known for silencing mobile elements such as retrotransposons (Weick and Miska, 2014). The common denominator between the different classes appears to be their regulatory roles in gene expression, however, while the function of many newly identified ncRNAs remains elusive, it cannot be excluded that some might not be functional.

The work included in this thesis only features miRNAs and, therefore, that will be the focus of the rest of the chapter. In the following sections I will describe how miRNAs are generated and what are their reported roles in development, disease and regeneration.

4.1 microRNA biogenesis

Among the different emerging classes of ncRNAs, one of the most rapidly growing is the class of miRNAs. They were first discovered in the early 1990s, when two studies reported the down-regulation of the gene lin-14, at a post-transcriptional level, by the gene lin-4 in *Caenorhabditis elegans*, suggesting a mechanism of RNA-RNA interaction through sequence complementarity (Lee, Feinbaum and Ambros, 1993; Wightman, Ha and Ruvkun, 1993). However, it was only when a second miRNA (*let-7*) was discovered years later, that miRNAs started to generate an increased interest in the scientific community (Pasquinelli et al., 2000; Reinhart et al., 2000). Soon, several groups started to observe a high degree of sequence conservation among different species and miRNAs were finally classified as a distinct class of regulatory RNAs (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001), opening the doors to a completely new research field. Currently, miRNAs are defined as endogenous and short non-coding RNA molecules (20-22 nts) that regulate gene expression post-transcriptionally. This regulation mainly occurs through the binding to the 3' untranslated region (3' UTR) of specific mRNA targets, leading to mRNA degradation and/or translational repression (Bushati and Cohen, 2007). Despite their specific binding to targets, some miRNAs can regulate hundreds of different transcripts (Lim et al., 2005). The increased attention given to miRNAs can be explained by their role as regulators of fundamental processes in cell fate determination and their involvement in several diseases, with the potential to be used as biomarkers (Bushati and Cohen, 2007; Pritchard, Cheng and Tewari, 2012).

Several components that are involved in the processing of miRNAs into a mature form have been identified so far and compose today the canonical miRNA Biogenesis pathway (Fig. 16) (Lin and Gregory, 2015). The formation of functional miRNAs starts with transcription into long primary miRNA transcripts (pri-miRNAs) by RNA polymerases II or III (Lee et al., 2004; Borchert, Lanier and Davidson, 2006), which is subsequently cleaved by the Microprocessor complex, which includes Drosha and DiGeorge syndrome critical region 8 (DGCR8), in the nucleus (Gregory et al., 2004). The resulting precursor hairpins (pre-miRNAs) are exported to the cytoplasm by Exportin-5 (Lund et al., 2004), where they are cleaved to their mature length by the RNase Dicer complex, forming miRNA duplexes (Hutvágner et al., 2001). One of the strands from these duplexes (guide strand) is then loaded together with Argonaute proteins (Ago) into the miRNA-induced silencing complex (miRISC), where it guides RISC to silence the respective target mRNAs (Schwarz et al., 2003; Chendrimada et al., 2005; Robb and Rana, 2007). In particular, the seed region, which refers to the nucleotides 2 to 8 that form the most conserved region of miRNAs, is important for target binding and therefore, it can be a useful feature for target prediction (Thomson, Bracken and Goodall, 2011). Target silencing can result in mRNA decay (through cleavage or deadenylation) or translational repression,

depending on how the miRNA binds to its target (Fabian and Sonenberg, 2012; Iwakawa and Tomari, 2015). Interestingly, certain miRNAs have also been shown to have the capacity to promote translation in quiescent cells (Vasudevan, Tong and Steitz, 2007).

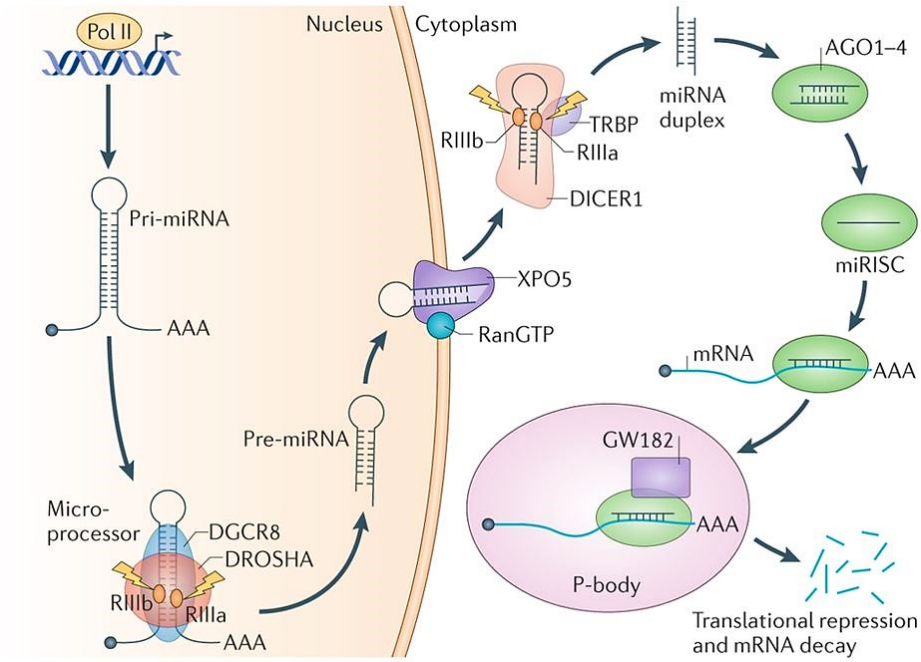

Figure 16: Overview of miRNA biogenesis pathway. MicroRNA (miRNA) genes are transcribed as primary mi RNAs (pri-miRNAs) by RNA polymerase II (Pol II) in the nucleus. The long pri-miRNAs are cleaved by Microprocessor, which includes DROSHA and DiGeorge syndrome critical region 8 (DGCR8), to produce the 60–70-nucleotide precursor miRNAs (pre-mi RNAs). The pre-mi RNAs are then exported from the nucleus to the cytoplasm by exportin 5 (XPO5) and further processed by DICER1, a ribonuclease III (RIII) enzyme that produces the mature miRNAs. One strand of the mature miRNA (the guide strand) is loaded into the miRNA-induced silencing complex (miRISC), which contains DICER1 and Argonaute (AGO) proteins, directs the miRISC to target mRNAs by sequence complementary binding and mediates gene suppression by targeted mRNA degradation and translational repression in processing bodies (P-bodies). TRBP-transactivation-responsive RNA-binding protein. (Reproduced with permission from: Lin and Gregory 2015).

In recent years, accumulating evidence from deep-sequencing studies indicates the existence of a high number of sequence variants in different tissues, both at the level of the precursors and the mature forms (isomirs) (Guo and Chen, 2014). Different sources for this variance have emerged such as RNA editing, nucleotide trimming or nucleotide addition (Neilsen, Goodall and Bracken, 2012; Vickers *et al.*, 2013). Interestingly, these modifications can work as a mechanism of regulating miRNA abundance through stabilization or targeting for degradation (Boele et al., 2014), suggesting a relevant biological role that might be tissue/cell-specific and that was previously overlooked.

4.2 microRNAs in development

As regulatory molecules that coordinate gene expression, miRNAs are implicated in a big range of biological processes that determine the fate of cells and tissues in an organism. In humans for instance, it has been estimated that more than 60% of all protein-coding genes are directly targeted and therefore regulated by miRNAs (Friedman et al., 2009). Although the level of repression induced by a single miRNA on a specific target is generally modest (Baek et al., 2008; Selbach et al., 2008), individual miRNAs can modulate hundreds of targets, often within the same biological pathways, which can have a great impact in overall cell behavior (Grün et al., 2005; Lim et al., 2005; Friedman et al., 2009). These particular features make miRNAs "the micromanagers of gene expression", as this fine-tuning of transcript levels is fundamental to determine and achieve homeostasis (Bartel and Chen, 2004). Unsurprisingly, abolishing miRNA biogenesis by knocking-out Dicer leads to early lethality in mice (Bernstein et al., 2003) and zebrafish (Wienholds et al., 2003), whereas DGCR8 deletion compromises proliferation and differentiation in mouse embryonic stem cells (Wang et al., 2007). On the other hand, the deletion of single miRNAs *in vivo* has no substantial effect on viability and does not produce obvious phenotypes in most cases (Park, Choi and McManus, 2010). This can be partly due to miRNA redundancy, by which other similar miRNAs might compensate for the ones missing (Fischer et al., 2015).

Nonetheless, loss-of-function studies have been an important tool to elucidate miRNA function, such as their major roles in coordinating the development of various organ systems. This is often accomplished through distinct sets of tissue-specific miRNAs that modulate development with unique temporal and spatial expression patterns, thus contributing to tissue identity (Sood *et al.*, 2006; Guo *et al.*, 2014). In the mammalian nervous system, miRNAs contribute for neuronal progenitor cells maturation into early neurons by inhibiting cell proliferation and subsequently inducing differentiation (Nishino *et al.*, 2008; Zhao *et al.*, 2009), or through brain-specific alternative splicing of pre-mRNAs (Makeyev *et al.*, 2007). In the developing heart, miRNAs have been mostly studied in the cardiomyocytes, where they control cardiac growth and differentiation by repressing multiple cell cycle regulators (Liu *et al.*, 2008; Porrello *et al.*, 2011), transcription factors (Zhao, Samal and Srivastava, 2005) and the tumor suppressor PTEN (Chen *et al.*, 2013). Other regulatory roles have been described for miRNAs in skeletal muscle myogenesis (Crist *et al.*, 2009), differentiation and stratification of skin (Lena *et al.*, 2008; Yi *et al.*, 2008), fetal lung branching (Bhaskaran *et al.*, 2009) and insulin secretion during pancreatic development (Poy *et al.*, 2004).

4.3 microRNAs in disease

MiRNAs have been found to be deregulated in a variety of diseases, which rapidly turned them into attractive potential therapeutic targets. Consistently with their functions in organogenesis, they are involved in cardiovascular disease (Olson, 2014), neurodegenerative disorders such as Alzheimer's or Parkinson's (Abe and Bonini, 2013) and numerous autoimmune diseases like diabetes, multiple sclerosis, rheumatoid arthritis and others (Singh *et al.*, 2013; Qu, Li and Fu, 2014). Additionally, miRNA expression profiles were found to be a reliable tool to identify origin and differentiation state of human tumors, whereas mRNA profiles proved to be highly inaccurate (Lu *et al.*, 2005). This important discovery highlighted their potential for cancer diagnosis and, since then, miRNAs have been widely studied in diverse cancer types, where they are typically divided between two groups: tumor suppressor and oncogenic miRNAs (oncomirs) (Catela Ivkovic *et al.*, 2017). Tumor suppressor miRNAs, which usually target oncogenes, are generally downregulated in a cancer context, whereas oncomirs modulate tumor suppressor genes and tend to be overexpressed in cancer.

Notably, miRNAs were also shown to be capable of regulating distant cells, without direct cell-to-cell interaction. These so called circulatory miRNAs are secreted from cells to the extracellular environment packaged into exosomes (Valadi *et al.*, 2007), high-density lipoprotein particles (Vickers *et al.*, 2011), or bound to protein complexes (Arroyo *et al.*, 2011), which makes them highly stable by providing protection against RNAse degradation (Cortez *et al.*, 2011). Furthermore, circulatory miRNAs were found to be present in bloodstream and other body fluids, which makes them easily accessible and raises the interest in their potential as disease biomarkers (Chen *et al.*, 2008; Mitchell *et al.*, 2008). In particular, this important feature can provide new insights into our understanding of cancer progression and development of metastases (Liu *et al.*, 2016), which might lead to improvements in our diagnosis and prognosis capacity.

Since early on, the strong link between miRNAs and disease has led to the design of multiple therapeutic strategies based on miRNAs. The current approaches are generally divided between the replacement of lost miRNAs, through the use of miRNA mimics, and inhibiting overexpressed miRNAs, with antagomiRs or miRNA sponges being the most common (van Rooij and Kauppinen, 2014). Despite promising candidates during preclinical research, most of those investigated so far have not progressed into advanced clinical evaluation, emphasizing the challenges of developing drugs from small molecules. On the other hand, a few drugs developed to target miRNAs involved in hepatitis C, hepatic cancer, lung cancer and T-cell lymphoma have already reached clinical trials (Catela Ivkovic *et al.*, 2017), thus holding hope for miRNA-based therapies in the future.

4.4 microRNAs in regeneration

Injury-induced regeneration is characterized by rapid changes in gene expression that require a strict regulation in order to trigger an adequate response. With the emergence of miRNAs as prominent agents in the regulation of gene expression in a tissue-specific fashion (Sood *et al.*, 2006; Guo *et al.*, 2014), it was not surprising that they have become interesting candidates in regeneration studies.

This assumption was first addressed in vertebrate regeneration in 2006 and 2007, by profiling the miRNA expression in newt lens and inner ear hair cells regeneration (Makarev et al., 2006; Tsonis et al., 2007). Without functional assays, these were the first studies reporting conserved microRNAs in the newt and correlating their altered expression with regeneration. Later on, this time through gain- and loss-of-function experiments, it was observed that the depletion of miR-133, via FGF signaling, is required for proper fin regeneration in zebrafish (Yin et al., 2008). Similar results were found a year later, where the inhibition of miR-196 impaired tail regeneration in axolotl, thus revealing an essential component for a precise gene regulation (Sehm et al., 2009). These studies have showed a high degree of evolutionary conservation among vertebrate species and opened the doors to several others that followed (Yu et al., 2011; Holman et al., 2012; Yin et al., 2012; Witman et al., 2013; Lepp and Carlone, 2014; Rajaram et al., 2014). Such interspecific similarities pose an important feature of the study of miRNAs as they can offer new opportunities in the identification of common regulatory networks that are required for tissue regeneration (King and Yin, 2016).

Despite their implication in regeneration and their functional role in appendage regeneration (Yin et al., 2008; Sehm et al., 2009; Holman et al., 2012), very little is known about their function in salamander limb regeneration, particularly in the context of muscle dedifferentiation. In mammals, some interesting reports have started to elucidate important roles of miRNAs in muscle regeneration. In skeletal muscle of adult mice, miR-206 was found to be essential for satellite cell differentiation during regeneration and to slow the progression of Duchenne muscular dystrophy (Liu *et al.*, 2012). In another study, a functional screening identified different miRNA candidates that induced cardiac regeneration and nearly full recovery of functional parameters in mice upon myocardial infarction (Eulalio *et al.*, 2012). Moreover, it has also been shown that miRNAs may regulate cellular reprogramming (Judson et al., 2009), which is consistent with the idea that miRNAs are likely to impinge on cellular dedifferentiation. Therefore, we have explored these possibilities in Paper II.

5. Centrosomes and post-mitotic state

Centrosomes are cell organelles that are present in most eukaryotic cells. They have been studied for over a century and are best known for their role as major microtubule organizing center (MTOC), which directs the microtubules to form the mitotic spindle during cell division (Schatten, Hueser and Chakrabarti, 2000). However, they play additional roles in cell motility, signaling, adhesion, coordination of protein trafficking and regulate cell polarity, usually microtubule-related functions (Conduit, Wainman and Raff, 2015; Werner, Pimenta-Marques and Bettencourt-Dias, 2017). Several studies have showed that a functional centrosome is required for cell cycle progression, as centrosome ablation (Hinchcliffe et al., 2001; Khodjakov and Rieder, 2001), knockdown of centrosome proteins (Srsen et al., 2006; Mikule et al., 2007) and chemical inhibition of centriole biogenesis (Wong et al., 2015), resulted in mitotic arrest. In this chapter I will cover the current knowledge regarding centrosome formation, their main roles in homeostasis and disease, and discuss potential functions in muscle differentiation and regeneration.

5.1 Centrosome assembly and function

Centrosomes are composed by two barrel-shaped microtubule structures, the centrioles, that are surrounded by a matrix of different proteins collectively called the pericentriolar material (PCM) (Fig. 17). Besides being integral part of the centrosome, centrioles are also necessary for assembly of cilia and flagella (Bettencourt-Dias, 2013). When a cell enters the cell cycle (G1-phase) it only has one centrosome, with two centrioles: the mother (older one) and the daughter centriole. During S-phase, these centrioles duplicate with two new centrioles forming orthogonally to the already existing ones. Towards the end of the interphase, when the cell is in G2-phase, the new daughter centrioles reach their maximum length and mature through the recruitment of components to the PCM, thus forming the centrosome. Once the cell enters mitosis, the two centrosomes separate and nucleate microtubules in order to assemble the mitotic spindle. Upon mitosis, both daughter cells have inherited one centrosome each, which will assure the continuation of the cell division process. If the cell does not proceed to a new cell cycle, then centrosome inactivation or ciliogenesis may occur (Fig. 18) (Conduit, Wainman and Raff, 2015; Werner, Pimenta-Marques and Bettencourt-Dias, 2017). In most quiescent cells, the mother centriole docks to the cell membrane and initiates the formation of a single cilium (Ishikawa and Marshall, 2011). This primary cilium is non-motile but behaves as a sensor for chemical and mechanical signals during vertebrate development (Goetz and Anderson, 2010).

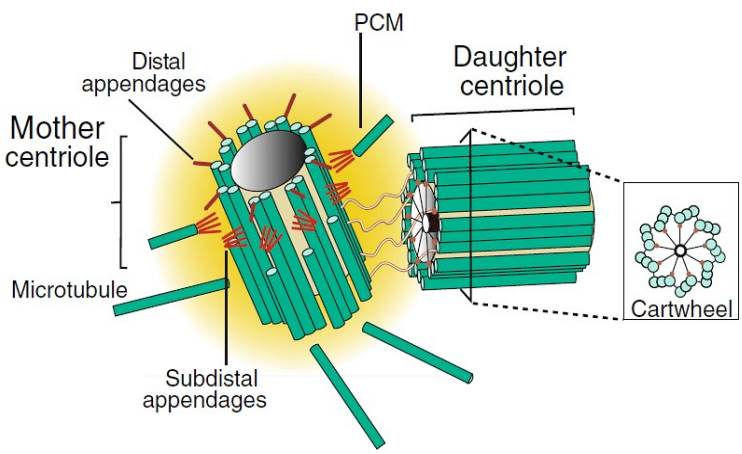

Figure 17: The structure of centrosomes. Each centrosome is composed of two centrioles (mother and daughter) and surrounded by a matrix of proteins called the pericentriolar material (PCM). The older centriole (mother) displays subdistal appendages, where microtubules are docked, and distal appendages, which are important for docking to the cell membrane. The canonical centriole has nine microtubule triplets and this nine-fold symmetry is in part provided by the cartwheel, one of the first centriole structures that is assembled. The cartwheel is then lost during centriole maturation. (From: Bettencourt-Dias, 2013)

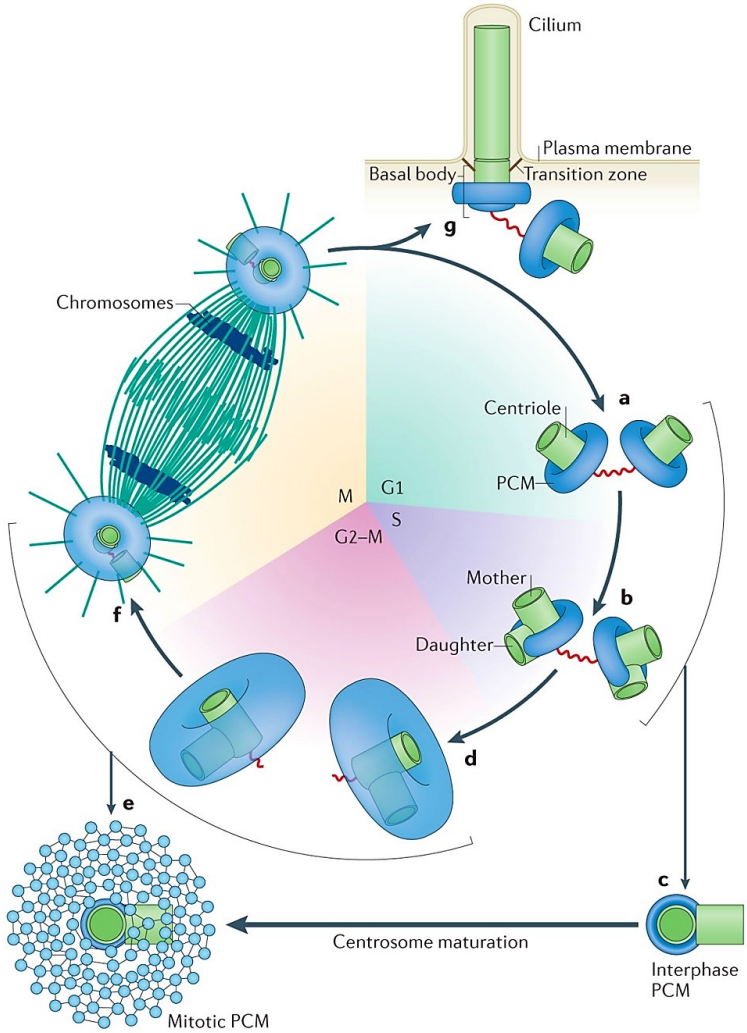

Figure 18: Centrosome duplication during the cell cycle. **a)** A 'newborn' cell in the G1 phase of the cell cycle usually contains two centrioles that are often joined together by a flexible linker (red). The centrioles can form centrosomes by organizing pericentriolar material (PCM) around themselves. **b)** The centrioles duplicate in the S phase, each forming a daughter centriole that is tightly apposed at to the original mother centriole in an 'engaged' configuration. Although the centrioles in most cells in G1–S organize very little PCM, this PCM is highly organized around the mother centriole (see part **c**). **d)** As cells enter mitosis (G2–M), the two pairs of centrioles start to move apart as the linkage between them is broken. The mother centrioles start to recruit much larger amounts of PCM, and this is thought to be organized by a 'scaffold' structure that assembles around the mother centrioles (see part **e**). **f)** The enlarged PCM allows the centrosomes to nucleate and organize many more microtubules, which then play an important part in assembling and positioning the mitotic spindle. As cells exit mitosis, the chromosomes segregate on the mitotic spindle and the mother and daughter centrioles disengage. **g)** In many animal cells that have exited the cell cycle, the centriole pair migrates to the cell surface, and the mother centriole forms a basal body from which a cilium extends. The cilium is known to have many important functions in cells, and cilium dysfunction is associated with many human pathologies. (Reproduced with permission from: Conduit *et al*., 2015)

In order to explain how centrosomes are assembled, it is fundamental to understand centriole assembly, as they are required for an efficient aggregation of the PCM (Bobinnec et al., 1998). Studies in worms (*C. elegans*) have been driving the progress in this field with several outstanding discoveries, which contributed to establish a whole set of core proteins, such as the Spindle assembly abnormal 4 (Sas4) (Kirkham et al., 2003), that are necessary for centriole assembly in all eukaryotes (Gönczy, 2012; Jana, Marteil and Bettencourt-Dias, 2014). The current challenge is to understand how these core components interact with each other and how they ensure that centrioles are properly assembled.

New evidence has emerged, through structural analysis of different proteins, which helped elucidating some of these mechanisms (Kitagawa et al., 2011; Van Breugel et al., 2011). When the new centrioles are formed after duplication, the subsequent assembly of the PCM starts. Although the levels of the PCM differ among cell types, they are overall reduced during interphase (Fig. 18) (Conduit, Wainman and Raff, 2015). In this stage, PCM only forms around the mother centriole, and displays a great level of organization (Lawo et al., 2012; Mennella et al., 2012). As cells progress towards mitosis and centrioles mature, the amount of PCM recruited by the centrioles highly increases (Mahen and Venkitaraman, 2012). This maturation process relies on phosphorylation events from different mitotic protein kinases such as Polo-like kinase 1 (Plk1) (Lane and Nigg, 1996). Subsequently, Plk1 was found to be involved in the recruitment of gamma-tubulin (γ-tub) and pericentrin (PCNT) (Casenghi et al., 2003; Haren, Stearns and Lüders, 2009), two important components of the PCM that mediate microtubule nucleation (Zimmerman et al., 2004).

5.2 Centrosome functions and conservation in evolution

During cell division, when the centrosome assumes the role of MTOC, it promotes chromosome segregation by assembling the mitotic spindle. Even though centrosomes have several other functions that go beyond cell division, these generally involve microtubules. They can coordinate protein trafficking by establishing microtubule tracks that motor proteins use to transport different cellular components across the cell. Additionally, they can modulate the transport speed and modify certain components before they proceed to their target location (Bettencourt-Dias, 2013; Royle, 2013). Centrosomes are also responsible for ensuring cell polarity in a variety of processes. In fertilization, centrosomes were found to be necessary for the union of the maternal and paternal genomes to occur in mouse, sea urchin (Schatten *et al.*, 1986) and *C.elegans* (Zonies *et al.*, 2010) eggs. In development, centrosome-based asymmetric cell divisions were also observed in mammals, with implications in human embryonic stem cell proliferation (Fuentealba *et al.*, 2008) and in the mouse developing neocortex (Wang *et al.*, 2009). As previously mentioned, centrioles in quiescent cells can switch between a centrosome to a cilia-forming basal body upon migration to the cell surface. Despite not being fully understood how this shift in animal cells occurs, as centrioles dictate centrosome properties, it is worth mentioning that the primary cilium is known to also play important functions during vertebrate development. Among other roles, it can work as a sensory organelle to a big range of stimuli originating from different signaling pathways (Goetz and Anderson, 2010; Bornens, 2012). For instance, this signaling was shown to be critical for survival and patterning of mouse embryos (Huangfu *et al.*, 2003). Interestingly, whereas some cell lineages do not form primary cilia, such as the immune system, which establishes transient immune synapses through centrosomes (Stinchcombe *et al.*, 2006), others like renal epithelia form primary cilia that play a critical role in the physiology of the kidney (Nauli *et al.*, 2003). What drives different cells to adopt distinct behaviors within the same organism remains unclear, but the extensive research in this field might soon contribute to a better understanding of this issue.

Centrosomes can be found across all the major eukaryotic groups and their conserved structure suggests that this could be an ancestral feature. While they are not present in all species, they were found in all animals studied and determined to be essential for their development (Debec, Sullivan and Bettencourt-Dias, 2010; Azimzadeh, 2014). However, there are some exceptional cases where they are dispensable. For instance, during early mouse embryogenesis, centrioles are naturally absent during the first cell-divisions of the blastomere (Szollosi, Calarco and Donahue, 1972). Additionally, when fruit flies have an homozygous mutation in the *sas4* gene, which is required for centriole duplication, this generates morphologically normal animals that are born without centrioles but die shortly after birth (Basto *et al.*, 2006). Surprisingly, planarians were recently found to be the first

animal species that completely lacks centrosomes, despite having centrioles to assemble cilia (Azimzadeh et al., 2012). This raises the question: are centrosomes strictly necessary for cell division? The current view is that it is not a general requirement since, besides being species-specific, it also depends on the cell type/tissue. While they might not be critical for cell division in some tissues, they are still indispensable in others, which seems to be the case in many organisms (Rodrigues-Martins *et al.*, 2008; Debec, Sullivan and Bettencourt-Dias, 2010). When looking at the distribution of centrosomes among eukaryotic organisms, a strong correlation can be observed between the presence of centrioles and cilia/flagella, whereas the presence of centrioles does not reflect existence of centrosomes in many cases. This observation indicates that the assembly of cilia/flagella might be the ancestral and most important function of the centrioles, rather than centrosome assembly (Bettencourt-Dias, 2013).

5.3 Centrosome-related diseases

Centrosome and centriole dysfunction have a broad range of implications in human health and have been linked to numerous diseases. Theodor Boveri, who extensively studied and named the centrosome (discovered earlier by Flemming and Van Beneden) (Schatten, Hueser and Chakrabarti, 2000; Delattre and Gönczy, 2004), was the first to link centrosome aberrations with disease more than 100 years ago. Strikingly, even before oncogenes and tumor suppressor genes were discovered, Boveri proposed that tumor formation resulted from loss of cell polarity and chromosome segregation abnormalities, further suggesting that centrosome structural defects were a major cause for these imbalances (Boveri, 2008). Boveri's influential work has provided the basis for many cancer studies that followed but, despite several observations that support an association between tumor formation and centrosome dysfunction, it is still a matter of debate whether these defects directly promote tumorigenesis or are simply a by-product of abnormal cell division (Bettencourt-Dias *et al.*, 2011; Nigg, Čajánek and Arquint, 2014).

More recently, his work was revived after being reported that knockdown of the tumor suppressor p53, downregulated in most mammalian tumors, led to multiple centrosomes in mouse fibroblasts (Fukasawa *et al.*, 1996). Additionally, it was found that dysregulation in centrosome duplication is common in a variety of tumors (Lingle *et al.*, 1998; Pihan *et al.*, 1998). These studies were fundamental to uncover a potential genetic link between centrosome aberrations and tumor development, which is still being investigated (Wu *et al.*, 2012; Nam and Van Deursen, 2014), further supporting Boveri's early predictions. Besides cancer, centrosome/centriole defects can also lead to neurodevelopmental disorders that might result in brain size anomalies and dwarfism, and ciliopathies (defects in cilia structure or function), which are associated with bronchitis, sinusitis, sperm immotility and changes in body symmetry (Bettencourt-Dias *et al.*, 2011; Nigg, Čajánek and Arquint, 2014).

5.4 Centrosomes and skeletal muscle

During the last decade, several studies have highlighted the prominent roles of centrosomes in asymmetric cell division, which is particularly relevant for stem cell maintenance and cell differentiation (Schatten and Sun, 2011). In addition, it has been known that centrosomes often become inactivated (i.e. lose their MTOC capacity) in animal cells that undergo differentiation. This is the case for neurons, epithelial cells and muscle cells, which establish alternative non-centrosomal MTOCs, thus resulting in a reorganization of the microtubule network (Lüders and Stearns, 2007; Jaworski, Hoogenraad and Akhmanova, 2008; Sanchez and Feldman, 2017). In particular, skeletal muscle, which is composed by long and post-mitotic multinucleated myofibers that derive from the fusion of mononucleated myoblasts (Dumont et al., 2015; Sampath, Sampath and Millay, 2018), switches from a radial network of microtubules to a parallel array of filaments along the extended cytoplasm (Fig. 19) (Warren, 1974; Tassin, Maro and Bornens, 1985; Connolly, Kiosses and Kalnins, 1986). Upon this transition, most of the microtubule nucleating capacity is taken over by the nuclear membrane (Tassin, Maro and Bornens, 1985; Bugnard, Zaal and Ralston, 2005; Srsen *et al.*, 2009).

Currently, the fate of centrosome components during myogenic differentiation is still an ongoing debate. This is due to the fact that earlier studies on the topic generally found that centrioles were missing or rarely seen in mature myotubes/myofibers (Przybylski, 1971; Connolly, Kiosses and Kalnins, 1986), while others reported they were relocated and not associated with nuclei once differentiation takes place (Warren, 1974; Tassin, Maro and Bornens, 1985). These conflicting observations could be partially explained by a combination of factors such as the different techniques used (electron microscopy and immunohistochemistry), species tested (muscle from chick embryos, frog tadpoles and human biopsies) and possible differences between differentiation stages of the cells

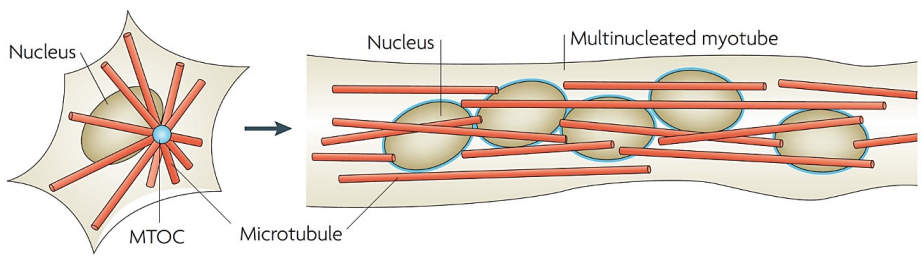

Figure 19: Microtubule reorganization during skeletal muscle differentiation. In mononucleated myoblasts (Left), the single microtubule-organizing center (MTOC) organizes a radial microtubule array. Upon differentiation into multinucleated myotubes (Right), the nuclear envelopes take over the role as MTOCs and the microtubule network is re-organized into a parallel distribution. (Reproduced with permission and adapted from: Lüders and Stearns 2007)

observed. Later on, several groups have tried to address this question and increasing evidence suggests that, upon differentiation, the centrosome disassembles with several of its components being redistributed to sites that nucleate microtubules, both in the nuclear envelope and cytoplasm (Musa *et al.*, 2003; Bugnard, Zaal and Ralston, 2005; Srsen *et al.*, 2009). Interestingly, a recent study in cardiac muscle reported that centrosome integrity was lost in adult mouse cardiomyocytes, contrary to what happened in adult zebrafish or newt cardiomyocytes, both of which with the capacity to proliferate (Zebrowski et al., 2015). This strongly suggests the existence of interspecific differences in centrosome maintenance mechanisms that we still do not understand completely. Additionally, it indicates that centrosome integrity might be associated with the capacity of certain cells to revert the post-mitotic state during regeneration events, as it happens in newts. Uncovering the molecular mechanisms that lead to centrosome loss/redistribution in mammals, but its maintenance in newts, could provide new insights to clarify existent roadblocks to regeneration. In the work presented in paper IV, we have explored these questions.

6. Present investigation

6.1 Aims

The general aim of this thesis was to investigate the events of muscle dedifferentiation during limb regeneration in newts and to gain a better understanding of how the process is regulated at the molecular level. In addition, we intended to provide new insights on how this regulation allows dedifferentiation to occur in newts while preventing it in mammals. In order to elucidate underlying regulatory mechanisms of salamander limb regeneration, we studied this process at different cellular and molecular levels such as: genomic sequences (**Paper I**), non-coding transcripts (**Paper II**), canonical signaling pathway proteins (**Paper III**) and intracellular organelles (**Paper IV**). In particular, the specific aims for each study were:

Θ Paper I – Sequencing of a salamander genome

We aimed to sequence and assemble the first salamander genome to understand how genome size and content relate to regeneration capacity.

Θ Paper II – microRNAs in limb regeneration

We attempted to identify microRNAs that regulate salamander limb regeneration and to determine their mechanisms of action.

Θ Paper III – BMP signaling in muscle dedifferentiation

The goal was to identify a long-sought serum component that induces newt myonuclei to reenter the cell cycle.

Θ Paper IV – Centrosomes in skeletal muscle

This study intended to clarify whether centrosomes are maintained upon skeletal muscle differentiation in mammals and salamanders, and to uncover a possible role for these cellular organelles in the process of dedifferentiation during salamander limb regeneration.

6.2 Paper I – Sequencing of a salamander genome

Salamanders have been used for a long time as a research model to study regeneration. However, in comparison to other model organisms, the lack of available genomic resources has hindered the progress in the development of new genetic tools that can help us to explore their remarkable regenerative capacities (Kumar and Simon, 2015). In this study we aimed to fill this gap by sequencing and assembling the first salamander genome. The genome of the Iberian-ribbed newt (*Pleurodeles waltl)* provided us with a platform to further investigate the genomic landscape of salamanders and uncover particular features of their giant genomes.

Θ Summary of the results

Salamanders have been reported to harbor some of the biggest genomes in the animal kingdom (Brockes, 2015). In particular, the genome size of the Iberian-ribbed newt was around 20 Gb, making it several times bigger than the human genome and one of the largest sequenced to date (Gregory, 2018). One of the main features of this large genome was the high abundance and diversity of repetitive elements such as class I and class II transposable elements. We found two thirds of this genome repetitive content to be formed by Gypsy retrotransposons and Harbinger transposons, which have strikingly expanded in salamanders. Moreover, the genome displayed a high frequency of two miRNA gene copies, specifically miR-427 and miR-93b, with the latter appearing to be a salamander-specific expansion. Interestingly, both of these miRNAs were found to contain a seed sequence that has been previously associated with embryonic stem-cell specificity and cell cycle regulation.

To assess whether they were regulated in regeneration, we mapped total RNA-seq reads from several different tissues, including regenerating limbs, and observed that the primary transcripts of both miRNAs were upregulated at 3 days post-amputation (dpa). A similar pattern was observed for some Harbinger and Gypsy elements, showing that these also responded to injury. Next we proceeded to investigate the presence of two transcription factors that are known to be important for early development (Pax3) and skeletal muscle regeneration (Pax7) (Epstein, 2000; Sandoval-Guzmán *et al.*, 2014; Buckingham and Relaix, 2015). Through manual curation of this gene family and *in situ* hybridization analysis, we detected the presence and expression of both of these paralog genes. To test their functionality, we generated knock-outs for each of those genes, through CRISPR/Cas9 technology. $Pax7^{-/-}$ mutants (F1) showed no impairment or defects in muscle development, whereas Pax3 mosaic-mutants (F0) died prematurely or developed anomalies such as the lack of muscle tissue in the limbs. Surprisingly, when we evaluated their limb regenerative capacity, we found the animals in both cases to regenerate normally. Whereas $Pax7^{-/-}$ gave rise to normal limbs with skeletal muscle, the

Pax3 mosaic-mutants regenerated limbs with appropriate morphology but without muscle.

Θ Discussion and future work

The sequencing and partial assembly of salamander genomes have been delayed due to the complexity and very large size of salamander genomes (Looso *et al.*, 2013; Looso, 2014). With this study, we have taken a step further by providing a new tool that can become instrumental in the study of salamander regeneration.

We have here elucidated that the expansion of specific transposable elements and miRNA genes were some of the features partially responsible for the large genome size of the Iberian-ribbed newt. Together with their regulation during limb regeneration, this data might help us discovering new mechanisms of salamander regeneration. In particular, in *Xenopus* and Zebrafish, miR-427 (known as miR-430 in Zebrafish) was shown to mediate clearing of maternal RNAs during development, when the embryos switch to express their own genes (Giraldez *et al.*, 2006; Lund *et al.*, 2009). We hypothesize that this miRNA might assume a similar role as a master regulator during limb regeneration, where it can stimulate cell plasticity, so that processes like dedifferentiation can occur. To test this hypothesis, functional studies through manipulation of the miRNA levels should be performed and evaluate whether there is an effect in limb regeneration.

Through our loss of function studies on Pax3 and Pax7, we were able to show that limb regeneration occurred in the absence of skeletal muscle and that, when muscle was absent, other tissues did not contribute for muscle formation after amputation. In addition, we observed that the importance of Pax3 for mammalian embryonic development (Epstein, 2000) was conserved in the Iberian-ribbed newt. Interestingly, other salamanders such as the Mexican axolotl lack the Pax3 gene (Nowoshilow *et al.*, 2018), which suggests that Pax7 (or other paralogues) might have additional functions in this species. Pax7 is a canonical marker for satellite cells, which are important to ensure muscle regeneration in mammals and salamanders (Buckingham and Relaix, 2015; Fei *et al.*, 2017). In the newt, however, dedifferentiation of fully mature myofibers also provides an important contribution to the regenerate (Sandoval-Guzmán *et al.*, 2014), which can potentially compensate for the lack of satellite cells. To answer this question, it is essential that we perform muscle lineage-tracing studies in the future.

Taken together, these data highlight how the information we gained from the salamander genome opens new possibilities for the study of limb regeneration. In the future, we hope this will be a valuable resource to develop new tools for functional studies, such as transgenic lines, making salamanders even more attractive model organisms.

6.3 Paper II – microRNAs in limb regeneration

MiRNAs have been shown to be important regulators of gene expression and to be associated with tissue-specificity (Sood *et al.*, 2006; Guo *et al.*, 2014). Moreover, several reports have elucidated miRNA roles in tissue regeneration (Yin *et al.*, 2008, 2012; Sehm *et al.*, 2009; Holman *et al.*, 2012; Witman *et al.*, 2013). Here, we profiled miRNAs that were regulated during newt limb regeneration and identified that regulation of miR-10b-5p was an important factor. In addition, by performing a cross-species study, we identified miRNAs with distinct regulation comparing newt and mammalian muscle cells, in an attempt to uncover miRNA-based differences that might explain their different dedifferentiation capacity.

Θ Summary of the results

To profile miRNAs during limb regeneration with special focus on skeletal muscle, we performed small RNA-seq from newt blastemas and stump muscle during limb regeneration. We found that many miRNAs were strongly regulated, with a group of few miRNAs representing the vast majority of all miRNAs in the sample. From these, miR-10b-5p stood out as the most abundant overall, which showed a lower expression level in the blastema compared to the stump or uninjured muscle. Interestingly, RNA-seq profiling of mammalian myotubes showed the opposite expression trend, in a cell based assay where they are stimulated to reverse their differentiated state. Therefore, we decided to explore miR-10b-5p further, to determine whether it played an important function in regeneration.

We then proceeded to characterize the expression levels and patterns in newt limb regeneration. Through qPCR analysis we observed that miR-10b-5p was downregulated upon amputation and gradually restored to normal levels after 18 days. Furthermore, *in situ* hybridization revealed this miRNA to be highly abundant in skeletal muscle tissue overall and in the 18 day-blastema. These data indicated that miR-10b-5p might be implicated in the regenerative response of the newt. To test it, we employed a mimic-based approach to overexpress miR-10b-5p during limb regeneration, when it was downregulated, resulting in a delay in the regenerative process. While none of the mimic injected limbs failed to regenerate to digit stages, half of them displayed shortening in limb size. This led us to hypothesize that miR-10b-5p could be coordinating cell proliferation. We then employed a BrdU assay, after mimic injection, to detect the proportion of cycling cells in the regenerating limbs. We estimated that the proportion of cells in S-phase was significantly reduced in limbs where mir-10b-5p was overexpressed, and that the size of the blastemas was substantially reduced. To assess whether this reduced growth was induced by programmed cell death, we performed a TUNEL assay in mimic-injected limbs, but found no differences in the level of cell death when compared to control limbs.

Θ Discussion and future work

Taken together, our data indicate that miR-10b-5p is downregulated during limb regeneration and that this downregulation is necessary to ensure a proper regenerative response. While overexpression of miR-10b-5p did not severely impaired regeneration, it was shown to affect the proportion of cycling cells and consequently blastema formation.

One of the main limitations we faced in the analysis of the small RNA-seq data was the absence of annotated miRNAs for the Red-spotted newt, the species in which the miRNA screen was performed. This prevented us from efficiently discriminating between the different isoforms and determining the mature sequence of miR-10b-5p. However, the characterization and functional experiments for miR-10b-5p were performed with Iberian-ribbed newts, whose genome and transcriptome we recently sequenced (**Paper I**). These resources can now help us to circumvent the previous problems and help us identifying the miRNA precursors.

It is still premature to establish a role for miR-10b-5p in regeneration, as we did not yet identify its acting mechanism during this process. Thus, the next logical step is to define candidate targets to be tested. We can predict this based on sequence complementarity between the seed sequence of miR-10b-5p and 3'UTR regions from the available transcriptome. Once putative candidates have been selected, we can measure their mRNA and protein levels, through qRT-PCR and western blot, and assess the downstream effects of miR-10b-5p manipulation. In addition, we can perform reporter assays to determine specific mRNA-miRNA interactions. This technique allows us to express a luciferase reporter-3'UTR construct and evaluate whether its expression is altered upon miRNA manipulation as a sign of direct binding.

In relation to the obtained mild phenotype, it is important to consider two points. First, the effects of injected mimics are transient and, therefore, the overexpression effect ceases once the mimic is cleared out of the system. To address this, we could electroporate a miRNA expressing construct, which would stably express the miRNA gene, and investigate how it affects regeneration. Second, it is plausible that certain compensatory mechanisms are in place, possibly through other miRNAs, which prevent a more severe phenotype. To explore this option, we can inject combinations of mimics that would overexpress different miRNAs simultaneously to examine its effects on limb regeneration.

In the future, to further elucidate a potential role of miR-10b-5p in muscle dedifferentiation specifically, one could use a transgenic line that allows for *in vivo* tracing of muscle cells during regeneration (as in **Paper IV**), so we can explore how the manipulation of miR-10b-5p affects this process in particular.

6.4 Paper III – BMP signaling in muscle dedifferentiation

Around 20 years ago, it was reported that serum proteases which regulate blood clotting seemed to be involved in initiation of regeneration (Tanaka *et al.*, 1997). This conclusion was based on the observation that when cultured newt myotubes were exposed to serum, they re-entered the cell cycle. Additionally, thrombin and plasmin were identified as important serum components which could trigger this response (Tanaka, Drechsel and Brockes, 1999). However, the downstream mechanisms and targets of these proteases have remained elusive. In this study, we provide new insights into these mechanisms by showing that members of the BMP signaling pathway are specifically targeted by thrombin and plasmin, and that these cleaved forms of BMPs are necessary to induce dedifferentiation *in vivo*.

Θ Summary of the results

In order to identify the S-phase re-entry inducing factor (SPRF), we employed a series of experiments which included column chromatography, SDS-PAGE and mass spectrometry. Through these analyses, we identified 34 major proteins such as BMP4, BMP5 and BMP7. Upon testing, only BMP4 induced a myotube response and correlated with S-phase re-entry activity. Moreover, we found BMP4 to be required and sufficient for S-phase re-entry. Interestingly, native BMP4-containing dimers from purification fractions were found to be much more potent in inducing cell-cycle re-entry than recombinant proteins.

Considering the involvement of serum proteases in activating the SPRF, we investigated whether BMPs were targeted directly by thrombin and plasmin. Treatments with these proteases resulted in a significantly higher activity of BMPs in inducing S-phase re-entry in myotubes, suggesting they were more potent when cleaved. By mapping the target sites on BMP4, we found multiple sites for both proteases, with thrombin cleaving the peptide with higher selectivity.

We then proceeded to test the role of BMP signaling in cell cycle re-entry of skeletal muscle cells *in vivo*. To do so, we induced the expression of dominant-negative BMP receptors specifically in newt skeletal muscle. Through an EdU assay to measure cycling cells during regeneration (EdU^+), we observed that expression of all the tested dominant-negative receptors induced a 20-25% reduction in the number of EdU^+ cells, showing that BMP signaling was implicated in regeneration-induced cell cycle re-entry *in vivo*. Further molecular analysis of BMP signaling, through a luciferase reporter assay, indicated that BMP signaling proceeded through the activation of downstream SMAD targets. In particular, dedifferentiated cells were positive for phosphorylated SMAD1/5/8, thus confirming SMAD activity.

Lastly, to examine the relevance of BMP protease activity for muscle cell-cycle re-entry *in vivo*, we induced the expression of a mutant BMP4 (ΔN-BMP4), which mimicked the cleaved BMP4 form, in the regenerating blastema. We found that, when compared to wildtype BMP, this cleaved-like BMP4 resulted in a higher cycling index of dedifferentiating muscle-derived progeny. Furthermore, when inhibitors of thrombin and plasmin were injected in regenerating limbs, this decreased the number of cells that incorporated EdU, showing that fewer cells were re-entering the cell cycle. Consistently, this effect was rescued by the expression of ΔN-BMP4 during limb regeneration.

Θ Discussion and future work

Muscle dedifferentiation is a known key step in newt limb regeneration (Sandoval-Guzmán *et al.*, 2014). In this report, we showed that BMPs were serum factors that stimulated cell cycle re-entry of differentiated newt skeletal myotubes and myofibers. This activity was triggered by serum proteases, including thrombin and plasmin, that specifically cleaved BMP, thus enhancing its activity. In this model, skeletal myofibers in intact limbs are not in contact with plasma BMPs which circulate within blood vessels. Upon limb amputation, the severed vessels leak plasma BMPs into the surrounding tissues, thus initiating the clotting cascade and the subsequent cleavage of BMPs. The myofiber-derived progeny is then exposed to and respond to the activated BMPs by re-entering the cell cycle.

It is important to note that while previous studies have implicated BMP signaling in early steps of amphibian limb regeneration (Beck *et al.*, 2006; Guimond *et al.*, 2010), due to the employed methodology, it was not possible to determine whether the negative effects on proliferation were directly mediated by BMPs. In our study, through cell-autonomous inhibition of BMP signaling, we confirmed that muscle-derived cell cycle re-entry was directly enhanced by components of the BMP pathway. Our data also suggested that this pathway might work in parallel with other pathways, as the inhibition of BMP signaling only led to a partial decrease in S-phase re-entry.

In summary, this work provides new insights into the underlying mechanisms of limb regeneration. Particularly, we gained a new understanding in how local injuries can induce the activation of the BMP signaling pathway and how this pathway acts directly at the cellular level to form the blastema from mature muscle fibers. Overall, this can have important implications for promoting a proliferative state during regeneration, which could open new possibilities within Regenerative medicine.

6.5 Paper IV – Centrosomes in skeletal muscle

Centrosomes are known to play different functions in the cell but best known for their role as MTOC during cell division, in which they assemble the mitotic spindle that segregates the chromosomes (Werner, Pimenta-Marques and Bettencourt-Dias, 2017). Differentiation of skeletal muscle, a tissue formed by multinucleated post-mitotic cells (Dumont *et al.*, 2015), induces modifications to the centrosomes. However, the type and extent of these modifications remains poorly understood, as available literature points towards centrosome degradation or relocalization as a consequence of mitotic arrest (Przybylski, 1971; Warren, 1974; Tassin, Maro and Bornens, 1985; Connolly, Kiosses and Kalnins, 1986). In this study, we clarify this issue and identify important differences between mammalian and salamander muscle. Further analyses are ongoing to uncover a possible role for these cellular organelles in the process of dedifferentiation during salamander limb regeneration.

Θ Summary of the results

To characterize centrosome modifications upon muscle differentiation, we isolated primary myoblasts from mice and cultured them in low serum conditions to induce differentiation. Through immunostainings, we thoroughly evaluated the presence of several essential components of the centrosome (both PCM and centriolar) during several stages of mammalian muscle differentiation. We observed that once myoblasts committed to differentiation, this triggered a gradual depletion of those components, starting with the disassembly of the PCM and followed by the centriolar proteins. In late stages of differentiation, all the analyzed components were absent, with the exception of the centriolar CEP135 and Centrobin (CNTB). Nevertheless, they were undetected in primary mouse myofibers through super resolution microscopy.

We then focused on the early stages of differentiation in order to identify the events that initiate centrosome disassembly. Here we observed that CNTB, typically associated exclusively with the daughter centriole (Ogungbenro *et al.*, 2018), was present in both centrioles once the cell withdrew from the cell cycle. This precluded the location shift of pericentrin (PCNT) from the centrosome to the nuclear envelope, a known step in muscle differentiation (Srsen *et al.*, 2009), showing that this might be one of the earliest events taking place when muscle cells commit to differentiation. Interestingly, no major differences were found in gene expression of different components, apart from the PCM component Plk1, which decreased in later stages of differentiation. By contrast, we detected an increase in its protein levels during the same period. Depletion of Plk1 through siRNA impaired the formation of late myotubes, suggesting that regulation of myogenesis in mouse might involve Plk1.

In opposition to the mouse, we found centrosomes to be maintained in newt primary myofibers, with no indication of loss of the analyzed components. Strikingly, through regrowth assays, we observed that these centrosomes were still active MTOCs as they polymerized microtubules in these terminally differentiated muscle cells. To test whether there was a correlation between centrosome maintenance and dedifferentiation, we employed linage tracing of dedifferentiated muscle cells, during salamander limb regeneration. We detected centrosomes in the majority of the myofiber-derived progeny, both in cycling and non-cycling cells, suggesting that the centrosome might not be required for blastema cell proliferation.

Θ Discussion and future work

This study provides evidence suggesting that the mechanisms of centrosome elimination which are present in the mouse are not conserved in the newt. Or, alternatively, newts might have evolved different ways of protecting their muscle tissue from centrosome disassembly. One of the current limitations is the fact that we relied exclusively on immunostainings to determine absence of given components. Ultimately, to be certain that the centriole structure is not present anymore, it would be important to confirm with other techniques such as electron microscopy. Additionally, it would be interesting to test whether culturing myotubes for longer periods would lead to the loss of the remaining components or if myotubes *in vitro* lack the capacity to acquire the same features of fully mature muscle fibers.

Although we could not establish a mechanism behind centrosome inactivation, our data suggest that CNTB relocation is an important event in the early stages of differentiation when centrosome loss starts. However, functional studies already performed, through siRNA-mediated knock-down of CNTB, have proven to be inconclusive. Plk1 on the other hand, which showed promising results as a regulator of late differentiation stages and has been implicated in zebrafish heart regeneration (Jopling *et al.*, 2010), is an interesting candidate to further explore differences between the two species.

Centrosomes were recently shown to be disengaged in mammalian cardiac muscle, in clear contrast to engaged centrosomes observed in other species with heart regeneration potential, such as newts and zebrafish (Zebrowski *et al.*, 2015). Similarly, we aim to determine whether this is recapitulated in skeletal muscle, which would further support a link to limb regeneration. Furthermore, one should investigate through lineage-tracing if artificial induction of mammalian muscle dedifferentiation requires *de novo* formation of a centrosome structure, or if they are not necessary factors in this process. Finally, as the centrosomes found in the newt muscle were shown to be functional, it would be informative to perturb centrosome function and assay how this would influence myogenic dedifferentiation.

7. Conclusions and future perspectives

The long and rich history of regenerative biology has generated several major discoveries in experimental research. Nevertheless, despite the initial optimism towards the possibility of regenerating lost body parts in humans, the progress in our understanding of regenerative phenomena has moved at a slower pace than previously anticipated. This strongly reflects how the complexity level of regeneration was largely underestimated. A good example to illustrate this point is the evolution of liver regeneration research. During the early studies of this process, the general conviction was that the action of an individual factor, either stimulator or inhibitor, was the key to understand liver regeneration. Later on, it became clear that this was not the case, as specific pathways started to be implicated in triggering the regenerative response (Fausto, Campbell and Riehle, 2006). Currently, the available literature in the field exposes a great deal of complexity, with a variety of receptors, growth factors, cytokines and signaling pathways being reported as important components in this context (Michalopoulos, 2013). This further supports the idea that tissue and organ regeneration involve an intricate network of interactions that ultimately leads to an appropriate response.

Nature displays a remarkable variety of means by which injured or lost tissues can be restored (Birnbaum and Alvarado, 2008; Brockes and Kumar, 2008). This is best reflected in cases where seemingly similar processes, such as salamander limb and tail regeneration, can occur in distinct ways. Despite specific similarities between these two processes, including epidermal wound healing and a dedifferentiation phase that contributes to blastema formation, detailed experimental and molecular analyses have revealed a number of fundamental differences. First, while the regenerating limb requires the presence of peripheral nerves (Singer, 1952), regenerating tail depends on the presence of the spinal cord (Holtzer, 1956). Second, the cell types that can switch cell lineage differs. In the limb, muscle-derived cells were observed in cartilage tissue (Lo, Allen and Brockes, 1993), while in the tail, it was reported that spinal cord cells can differentiate into muscle and cartilage (Echeverri and Tanaka, 2002). Third, even though the ablation of muscle and skin performed unilaterally over half the limb circumference gives rise to half limbs (Goss, 1957), the same procedure in the tail causes no regeneration defects (Dinsmore, 1981).

Whereas the great complexity of regenerative phenomena can be discouraging for some researchers, a more optimistic perspective would highlight the immense possibilities that remain unexplored in regeneration research today. Moreover, we now have an array of new tools at our disposal to study them. In this thesis I have described how the field of regenerative biology has progressed over the years and, in my view, the most important milestones that have been achieved. I have focused on salamander research to greater

detail mainly for being the central topic of this work, but also to emphasize how this model organism has been instrumental in leading to many of those major discoveries. Furthermore, the studies included in this thesis have led to new insights into the mechanisms that govern salamander limb regeneration and, in particular, that are involved in skeletal muscle dedifferentiation. Ultimately, while many questions remained unanswered and new ones have emerged, we have been able to:

1) Identify unique features of a gigantic newt genome, namely the expansion of transposable elements and miRNAs. Moreover, we found an association between these features and limb regeneration. This study also equipped the field with a new resource that can be valuable for future regeneration studies (**Paper I**);

2) Profile miRNAs that are regulated during newt limb regeneration, and determine that an appropriate regulation of miR-10b-5p is important for blastema formation and for limb regeneration to occur at a normal rate (**Paper II**);

3) Establish a molecular mechanism of how limb regeneration is initiated upon injury. More specifically, we identified components of the BMP signaling pathway that are essential to promote myofiber dedifferentiation and subsequent blastema formation (**Paper III**);

4) Demonstrate that centrosome elimination that follows skeletal muscle differentiation in mammals, does not occur in newt skeletal muscle. We observed that newts maintain centrosomes in fully mature myofibers and that these are still functional MTOCs. This might have implications for cell-cycle re-entry which takes place upon myofiber dedifferentiation and, consistently, we found a majority of muscle-derived cells in the newt regenerating limb to contain centrosomes (**Paper IV**).

These and previous studies emphasize the importance of studying less conventional organisms, such as salamanders and zebrafish, to understand natural phenomena like regeneration. As mammals do not display a similar level of regenerative capacity, the lessons we learn from these alternative models might become essential for the development of future therapies in regenerative medicine.

8. Acknowledgements

It goes without saying that in science, just as in life, very few things can be accomplished alone. I am fortunate to be surrounded by many people who have supported and lifted me during these challenging years, both in my daily work and in my daily life, bringing me joy and pushing me towards the finish line. Here I hope to pay tribute to all of those who, in one way or another, have been important in this quest.

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♦ The Lab ♦

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storm, that everything will work out fine in the end. It seems simple but sometimes is hard to remember. Speaking of storm, I hope that Aston Villa will soon be ready to leave the intensive care unit. Goyalla, how is life? It was interesting to see your evolution from shy Shahul to (usually shirtless) party animal Shahul. I enjoyed every single argument we had over lunch, mostly about useless things. Thank you for introducing me to the Holi festival and to show me that indian food can be tasty without melting my throat. Laure, my (always organized) desk and lab bench neighbor. Nobody else shared my taste for indie music, so it was great to have you around. Even if that meant having to use up some energy to unplug your laptop every day. Tiago, we can all agree you were the second best Portuguese in the lab. Or in other words… you were the second most awesome. You are the only one of us with a pathological addiction for building snowmen. I'm not sure how that happened but I'm surprised you haven't yet discovered an app that can make them for you. I admire your obsession for detail in all the things you do and I am genuinely looking forward to see your future unfolding. Speaking of future, it is rapidly approaching the time in which I start remembering and worrying about your paparazzi tendencies. But remember, I know where you live and the PhD gave me some practice with sharp tools! Alberto, the salamander whisperer, I think it is a good sign that, even though we worked in the same lab, most of my memories are from parties (including joint birthdays), snowboarding, barbecues and other fun activities we enjoyed together. "La primavera trompetera ya llego!" ♪♫. But the most important lesson is to never forget to bring your backpack! Please send my greetings to Argos (and perhaps a biscuit).

Iv, I am happy the only pandemic outbreak we had to handle as scientists was during a board game. That was a fun night! I will remember your history lessons in shiny wigs and your team building spirit. Thank you for correcting my English whenever was necessary and best of luck for the remainder of your own journey. Ahmed, your excitement for science is contagious, thank you for showing me the importance of keeping a positive mindset. Justyna, even though it was impossible to drag you to the weekly after-work beer, I wish you would have stayed with us a bit longer, so you could keep telling me how you thought I looked like a post-doc. Eric, I am happy you always kept the salamanders happy by giving them food and music. As some of them came from France, I'm sure they appreciated the French radio station. Anoop, I have a hard time imagining how this thesis could have been finished without your help, knowledge and wisdom! Your contribution was essential to finish many experiments, but I am also incredibly grateful for all the advice, discussions about regeneration and proofreading! You are like the encyclopedia of regeneration and I have learnt a lot from you since you joined the lab. Zeyu, you are a hard-worker and I am sure you will succeed in the journey you are starting now. Good luck! Alex, keep taking good care of the salamanders and remember they also enjoy a good mojito on fridays after work. Elayia, the most recent member. You slowly started helping me with a few things here and there, but soon became a critical part of the team. Thank you for all your efforts, especially after I left the lab! I hope you enjoy your time in Stockholm, and don't worry about the darkness, it always passes.

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♦ CMB ♦

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♦ CMB Pub ♦

So many good memories with this one. It always gave me a chance to catch up with many "long time no see" friends. I believe we made CMB the coolest department in town and of course this was only possible with an amazing crew behind it. Helena C., whatever I write will never really do you justice so, instead, I will just wave my arms in the air like an inflatable arm-flailing tube man while writing this (not easy I can tell you). Now that you have visualized that in your mind, let me just thank you for all the laughter over the years! And for your pants. Thibaud, the best Odlaw (Waldo villain) ever entering in CMB. I still owe you some Hela cells, let me know if you ever want them back. Isabelle, the queen of dressing up who would totally come to work in full costume if that would not attract weird looks. Because people should definitely fight for their right to wear leopard underwear on top of their clothes! As a member of your admission committee, I look forward to your defense. No pressure. Pedro V., what to say? The only thing that comes to my mind is "Où est ton papa? Dis-moi où est ton papa? Papaoutai! Papaoutai!" ♪♫. Meeting the famous Veliça from FCUL in Stockholm, was a powerful reminder of how small this world is. Keep it up with the fantastic work with pedromics, because I feel that time flies when you are having a pun. Thank you for the awesome job with the cover (it's my favorite part of the thesis). Milind, the parties at your place were so much fun! I just hope I didn't make a mess with my mushrooms and fire balls. Yildiz (or kırmızı), I miss exchanging useless words in each other languages. The pubs were definitely not the same without you hijacking the playlist, good old times! Let me know if you ever want to go back to relax on a black ski slope. Anna, nobody plays the tambourines in karaoke like you do. But I also don't think anyone else sees the point in doing so. Thank you for teaching me 오리 고기, I should now be able to survive in South Korea without starving. Fosco, after you and Davide left, my foosball skills severely suffered from the lack of training during working hours. Please come back so we can fix that before I start my next job. Steffi, thank you for explaining me the differences between Bulgarians and Macedonians so eloquently.

♦ KI bubble ♦

To my remaining KI friends from other departments who wished they worked in CMB (even if they didn't acknowledge it). Alča, I hope I can one day climb mountains half as high as the ones you climb. If you keep aiming that high, you seriously risk achieving great things! Teresa F., you're a fighter and I'm sure your future has some good surprises waiting for you. I always enjoyed your friendship and the nice chats we had, except the time we went to watch the final of the Europa League. Damn you Sevilla! Raquel V., former short-term university teacher and TV star! I am still waiting to see you paddling on a dragon boat. Thank you for dropping by CMB once in a while for a quick catch up. Theresa M., it is amazing to see you having so much initiative in all these different events, I wish there would be more like you. See you soon in industry! Nina, I lost count to all the ski slopes we covered but it was a lot of fun. Let me know when you are ready for the next riddle, I have not used them all! Thank you for showing me that Opera is not as bad as I thought. Mei Ling, thank you for showing me Peter Russell, it was fun to see you laughing that hard. I feel the time for a new chapter has arrived and hope things will work out well from now on. I'm cheering for you! Greg, it was fun to hang out with you in Bodrum and in the parties that followed. I guess I managed to keep all these memories because, unlike Tiago, I remember your face. Mirjam, when are we going to 3D-print some more stuff? Beep me up when you have time, I could use a shield for my defense. Anneliese, thank you for bringing me into the scientific illustration world. Looking forward to the next gatherings!

To the Winter conference gang, this was one of the coolest initiatives I had the chance to part of during my PhD. It was amazing to see how the whole event evolved and I hope others will now keep carrying the torch for many years to come. Sebastian, the fearless captain of this enterprise. Little did we know, after the famous ping pong nights in Bodrum, all parties we would enjoy together. It was sad to see you leave but I hope everything works out well in Japan! Mat, you are present in most of my memories from KI. That basically means a lot of conversations about autophagy. You are one of the coolest I had the chance to hang out with and, now that you stopped fooling around with your defense date just for us to have a joint party, guess who will be the DJ in my party? (Hint: someone with a doctoral degree). Burcu, I believe I won't ever meet anyone else who enjoys listening to horror movies while pipetting in the lab. No matter what mood or time of the day, you are always someone fun to be around and your happiness spreads easily. Antonio, you had to leave but your killer moves on the dance floor stayed with us. All the best for the med school journey! Caroline, you were one of the core elements from the start and, in the process, you became a great friend. Always caring, happy to meet up and also checking on me to make sure I was surviving during crazy periods. Now that I am in Huddinge, it's time to make up for the lost time. Veronika, we met long time ago during a very frosty night in Solvik, in the middle of that majestic winter wonderland. I am happy we had the chance to enjoy a lot of other events together after that. But please stay safe, no point in insisting on a career in extreme sports anymore. Sandra, my favourite Menorcan! The one who just managed to meet everyone in KI in a very short time. Always amazed me! I'm happy we upgraded it to proper friends after just meeting through Ana. Sander it was great to have you onboard for the memorable edition in Åre! Tina, it was awesome to see you taking over the last edition of the conference with such enthusiasm!

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♦ Home, sweet Lappis ♦

After living in Lappis for so long, this certainly represents a big part of what Stockholm is to me. Over the years, the family I formed there kept growing. Fra, I am having withdrawal symptoms from not hearing about the twins for so long. Thank you for introducing me to Taralli (life changing!) and for informing me about all things that come from Kuneo. I wish that winter night we walked all the way to lappis through the snow could be repeated, it was incredible! Daniele, the man of the woods! it is awesome to finally have you with us. I wish I would see you more often but you guys are always kayaking around the world. Davide, I could thank you for all the qPCR tips, but what I remember more often is all the exercise we did together. Foosball games, pushing shopping carts full of hydrating beverages and carrying furniture around Lappis, that's a lot of arm workout. A reunion Finland shall happen soon! Justyna K., it was unfortunate you were not in Stockholm for longer because it was great to have you around. And I don't say it just because of all the vodka tasting. Please tell Giulia she is welcome to randomly call me again whenever she feels like. Jöelle, the best Cruella de Vil I have seen! Even though I have only seen two and one was a cartoon. I did not forget the promised trip to Giethoorn, but it is hard to handle the long bucket list. Nico, the brother from another mother. Our dinners are one of my best memories in Stockholm. Not so much for the food portion size because that was usually non-sense, but mostly for all the laughter (≥ 80% of the time making fun of Elisa) and for the occasional pragmatic advice. Also, I think it is time for you to watch The Bridge. Caro, it was sad when you left us to pursue a career in the weather agency, especially because I miss arancini and you never have good inside information about the weather anyways. Please tell your dad to call me Dr. Leon from now on. Elisa Curry Kahlúa, (or Princess of Lebanon), thank you for showing me that members of the royalty can also be skilled in the kitchen. However, I won't say you make the best tiramisu because Francesca might read this one day. Simone, my second favourite Simo. I am almost a free man again, which means it is time to go to the vinyl store! Alina, it was such a fun time when you were around, with the corridor dinners and all the big hiking plans that never took off after the first trip. Thank you for hosting me in Mainz and for showing me that Germany can also have good wine! It is your turn to come and visit. Ozan, yo man! It is time to switch the laptop for the racket, let's go back to the court. I believe that I will feel lighter after the defense and so I will have bigger chances of trashing you. But maybe that is just me daydreaming.

♦ Stockholm syndrome ♦

To all my Portuguese fellow emigrants Nuno, Andreia, Diogo, Joana, Catarina, Cristina, Cláudia, Moutinho, Antero, Filipa, and Mariana who also deeply bonded with the city that kept us in captivity within its borders, thank you for all the memorable moments over the years. Antero, és um tipo porreiro, mas estaria a mentir se dissesse que não gostei de te encher a cara com pó de várias cores no Karolinska. Andreia (ou meia-leca), tentei impingir-te o snowboard e não ficaste convencida. A ver se corre melhor com o mergulho. Pelo menos é mais difícil caíres. Diogo, (ou Frouto) quando é que vamos aos bifes outra vez? Estás sempre pela Ásia hoje em dia, não fica muito em caminho. Cláudia, Acho que está na hora de ir à Costa Rica, estamos à espera de quê? Moutinho, está na hora de desistir da carreira no futebol, tendo em conta os teus joelhos acabados e pés quadrados. Por outro lado, a carreira de palhaço continua a ser uma opção bastante válida em que todos te reconhem um grande potencial. Erik, the infiltrated swede who is by now more Portuguese than many of us. At least when it comes to the waves. Thank you for all the support with sound systems, moving apartments and tie knot tutorials.

Leonor, foi óptimo ter-te aqui "ao lado" mesmo que não nos tenhamos encontrado tanto quanto gostaria. Mesmo assim, curti à grande todos os Valborgs, aniversários, halloweens e restantes eventos. Obrigado por me teres introduzido ao mundo do Douglas Adams! Joana, confesso que já não me faz muito sentido continuar a chamar-te sueca. Mostraste-me a cultura deste país, tentaste ensinar-me a língua e levaste-me a andar sobre um lago congelado pela primeira vez. Apesar da distância actual e de viveres desconectada das redes socias, fico feliz por irmos mantendo o contacto. Espero ver-te em breve.

♦ The Core ♦

From the Croatian trip to all the dinners, parties and other gatherings, thank you for all the pretty epic memories. Just wait for the next episodes because, as always, I might have some new stories to tell. Hanna (or nagy), our favorite Balkan! Except when you drove us around during the holidays, that was a bit too intense. I can't really say there is a time in Stockholm before you, because we met at the very beginning. And it has been a hell of a ride! One of my proudest moments was to convince you to try tuna. Which says a lot about how picky you are. Raquel T., the most badass from Barreiro! Thank you for all the courier services over the years that brought me my suit, my ID card and packages of rice! If only you would know that making me a Pastel de Nata offer would have triggered so many requests, you would have reconsidered your actions. November is coming, so it is time for film festival! Garcia, thank you for vacating the room that saved me from the bed bugs. That was a small step for you, but a big step for my sanity! When are we gonna go snorkeling (like hell)? I should have more time after the defense. If you want, you can bring your sloth, no questions asked. But let's not talk about Frequencies again. Bettina, Get a grape! I'm not sure I will forgive you for leaving us like this, you never warned us that it was for good. We only adopted Viktoria to the group as a temporary solution, but now it has been so long, I don't even remember how your pineapple-shaped head looks like anymore. Just come back already. Viktoria, Don't read the one above, it's not actually true. We adopted you because it is fun trying to guess every time we meet what colour your hair will be. I hope you learnt your lesson regarding passports. It is nice to live on the edge sometimes, but no need to bring everyone along.

♦ Last, but certainly not least ♦

To all my friends from home who always "kept me close" while I was gone and made me look forward to our summer and Christmas reunions. Ao grupo dos Lóris Gordos: Pipas, Euclides, Zaca, Pimenta, Bruna, Petrov, Lia, Rita Martins, Maria, Unas, David, obrigado por estarem sempre na mesma, parvos como é preciso. Petrov, fazes falta cá na Suécia. Já não tenho quem acampe no meu sofá ou que queira fazer maratonas de Saw comigo. Joaninha das Neves e Rita Borba, não se esqueçam que me devem um Oktoberfest.

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