

MOLECULAR CHARACTERIZATION OF EARLY DEDIFFERENTIATION IN NEWT FORELIMB REGENERATION

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ABSTRACT

Newts have the incredible ability to regenerate many different organs and tissues as adults, including the limbs. Limb regeneration occurs via the dedifferentiation of stump tissue and the formation of a blastema, which provides the majority of cells for the regenerate. Despite all that we have learned about dedifferentiation and blastema formation, the cellular and molecular mechanisms underlying these processes are still poorly understood.

We used representational difference analysis (RDA) to identify genes involved in the early dedifferentiation process in newt forelimb regeneration. Our analysis identified approximately 410 unique genes that were differentially regulated during this process. Microarray analysis was used to determine the expression profile of these genes throughout limb and tail regeneration. We used quantitative PCR (qPCR) to validate the expression of a subset of these genes [*β -catenin*, *wntless*, *dapper*, *thymosin- β 4* (*T β 4*), and *thymosin- β 10/15* (*T β 10/15*)] in regenerating limb and tail tissue, as well as in differentiating newt myoblasts. We also verified the expression of these genes in the regenerating newt limb using immunohistochemistry (IHC) and *in situ* hybridization (ISH). Finally, we performed a functional analysis on *β -catenin*, *wntless*, *dapper*, and *T β 4* by overexpressing these genes in mouse myoblasts to examine their effects on differentiation and potential roles in dedifferentiation.

Quantitative PCR verified the expression of *β -catenin*, *wntless*, *dapper*, and *T β 4* during limb regeneration and IHC/ISH localized the β -catenin and T β 4 proteins to the blastema during regeneration. *T β 10/15* was shown by qPCR to be expressed in the tail during regeneration. Overexpression of newt *β -catenin*, *wntless*, *dapper*, and *T β 4* in

mouse myoblasts showed that each of these genes has an inhibitory effect on the differentiation of myoblasts into myotubes and, therefore, may play a role in promoting or maintaining the dedifferentiated state.

Our work has identified a large number of genes with potential roles in regulating the dedifferentiation process during newt forelimb regeneration. We have also laid a framework from which much more work can be done by drawing on the genes we have identified and the microarray data, which indicate ideal follow-up candidates. Our analysis of specific genes has also increased our understanding of the molecular events occurring during the dedifferentiation process in the regenerating newt limb.

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LIST OF ABBREVIATIONS

ADM – amphibian differentiation media

AEC – apical epithelial cap

AGM – amphibian growth media

AIFM – apoptosis inducing factor

ANOVA – analysis of variance

ANXA – annexin

AP – alkaline phosphatase

ARF – alternative reading frame

BCIP – 5-bromo-4-chloro-3'-indolylphosphate

BLAST – basic local alignment search tool

BLASTP – basic local alignment search tool (protein)

BMP – bone morphogenetic protein

BSA – bovine serum albumin

cDNA – complementary DNA

CDS – coding sequence

CMB – confluent myoblasts

C_T – threshold cycle

DAPI – 4',6-diamidino-2-phenylindole

DIG-dUTP – digoxigenin-11-deoxyuridine triphosphate

Dkk – dickkopf

DM – differentiation media

DMSO – dimethyl sulfoxide

DNA – deoxyribonucleic acid

ECM – extracellular matrix

EDTA – ethylenediaminetetraacetic acid

EST – expressed sequence tag

FC – fold change

GAPDH – glyceraldehyde 3-phosphate dehydrogenase

GFP – green fluorescent protein

GM – growth media

GPCR – G-protein coupled receptor

H & E – haematoxylin and eosin

HPRT – hypoxanthine-guanine phosphoribosyltransferase

HSP – heat shock protein

IHC – immunohistochemistry

IPTG – isopropyl β -D-1-thiogalactopyranoside

ISH – *in situ* hybridization

KRT – keratin

LB – lysogeny broth/Luria-Bertani

MABT – maleic acid buffer with tween-20

MHC – myosin heavy chain

MMP – matrix metalloproteinase

MRF – myogenic regulatory factor

MRL – Murphy Roths Large

mRNA – messenger RNA

MT – myotubes

NADH – nicotinamide adenine dinucleotide dehydrogenase

nAG – newt anterior gradient

NBT – nitro-blue tetrazolium

OCT – Optimal Cutting Temperature compound

PAGE – polyacrylimide gel electrophoresis

PBS – phosphate-buffered saline

PBS-T – PBS with tris

PCR – polymerase chain reaction

PEC – pigmented epithelial cell

PFA – paraformaldehyde

qPCR – quantitative PCR

RACE – rapid amplification of cDNA ends

Rb - retinoblastoma

RDA – representational difference analysis

RIPA – radioimmunoprecipitation assay

RLM-RACE – RNA ligase mediated RACE

RNA – ribonucleic acid

RNAi – RNA interference

ROS – reactive oxygen species

RPM – revolutions per minute

RT-PCR – reverse transcriptase-PCR

SCMB – sub-confluent myoblasts

SDS – sodium dodecyl sulphate

SP – side population

TAP – tobacco acid pyrophosphatase

T β – thymosin-beta

TBS – tris-buffered saline

TdT – terminal deoxynucleotidyl transferase

TE – tris-EDTA

TIMP – tissue inhibitor of metalloproteinase

UTR – untranslated region

VEGF – vascular endothelial growth factor

Wnt – *wingless* and *integration 1*

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INTRODUCTION

1.1 – Regeneration in the Animal Kingdom

Regeneration, a phenomenon observed to different extents throughout the animal kingdom, is commonly defined as the ability to recreate or repair tissues, organs, or body parts that have been lost or damaged. Regeneration is achieved in many different ways by the various species possessing this ability, including regeneration via stem cells, compensatory growth, morphallaxis, and epimorphosis, or a combination of these processes (Agata *et al.*, 2007). Some organisms, like flatworms (planarians), are able to regenerate an entire animal from just a fraction of the original through a population of stem cells, called neoblasts (Alvarado, 2007; Rossi *et al.*, 2008). Many vertebrate species are also known to maintain different populations of adult stem cells, which provide them with a source for regeneration and tissue homeostasis in tissues such as the digestive tract, skin, muscle, and blood (Kørbling and Estrov, 2003; Prentice, 2004). Compensatory growth is regeneration of a tissue through cellular proliferation alone. It is best exemplified by liver regeneration in mammals, where, following the removal of a portion of the liver, the remaining organ grows in size (via cellular proliferation) to compensate for the missing part (Duncan *et al.*, 2009). Morphallaxis is best exemplified by the *Hydra*, a cnidarian genus containing various species that regenerate by remodeling the remaining tissue following injury (without cellular proliferation) to create new organisms (Agata *et al.*, 2007). Finally, epimorphosis is a method of regeneration which includes dedifferentiation (the reprogramming of differentiated cells and their return to a

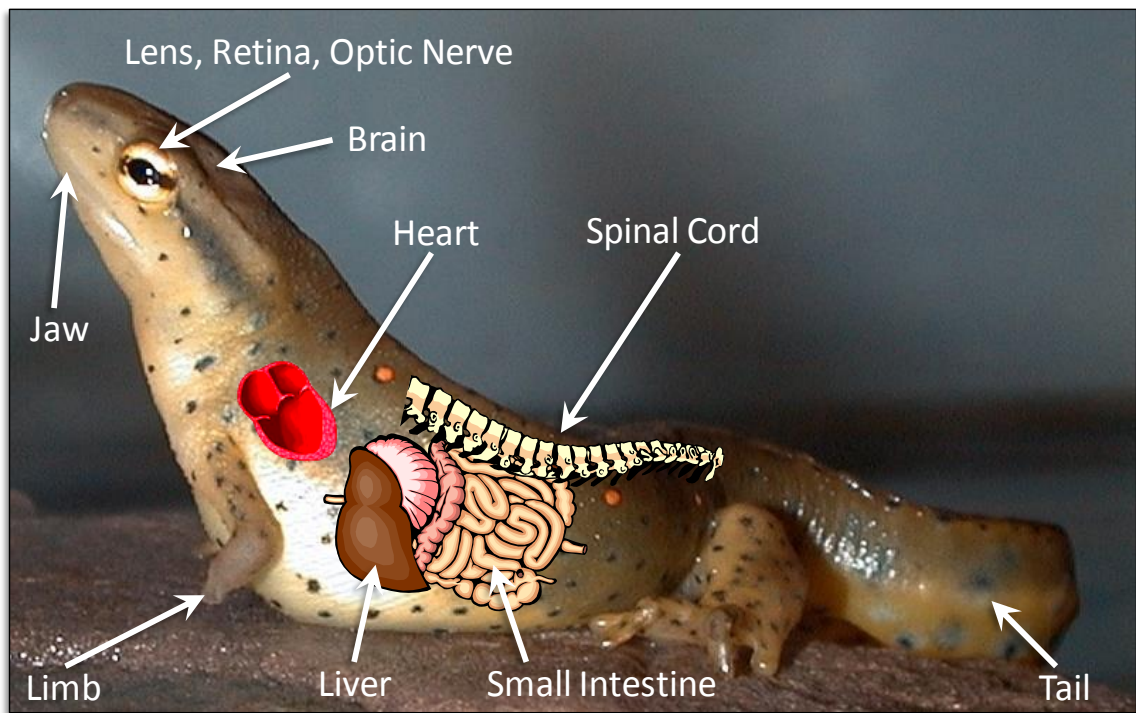
more progenitor-like state) along with cellular proliferation (Agata *et al.*, 2007). This type of regeneration is exhibited by fish and amphibians.

In general, more ancestral organisms (*e.g.*, *Hydra* and planarians) possess greater regenerative abilities than more derived organisms (*e.g.*, arthropods, fish, amphibians, reptiles, and mammals). For example, both *Hydra* and planarians can regenerate an entire animal from a small fraction of the original (Agata *et al.*, 2007), while many species of insect, fish, and amphibians possess the ability to regenerate their entire limbs/fins (Nakamura *et al.*, 2008; Nye *et al.*, 2003; Slack *et al.*, 2004; Tal *et al.*, 2010). However, in reptiles, only some species possess the ability to regenerate the tail and in mammals, mice and humans can regenerate the digit tips, but only as juveniles (Bateman and Fleming, 2009; Clause and Capaldi, 2006; Illingworth, 1974; Lehoczky *et al.*, 2011).

1.2 – Regeneration Among Vertebrates

Within vertebrates, specifically, there are a number of species that exhibit extensive regenerative abilities. For example, zebrafish (*Danio rerio*) can regenerate their fins, spinal cord, and heart tissue; frogs (*e.g.*, *Xenopus sp.*) can regenerate their limbs and tails, but only during their larval life stage (*i.e.*, as tadpoles); and urodele amphibians (*i.e.*, newts and salamanders) can regenerate many different tissues and organs. In fact, one of the most dramatic examples of regenerative ability, at least among adult vertebrates, is the eastern, red-spotted newt, *Notophthalmus viridescens*. This species has the ability to regenerate both the upper and lower jaw, eye structures like the lens, retina, and optic nerve, parts of the central nervous system (including the spinal cord), a portion of the heart, intestine, and liver, and even the entire tail and limbs ([Fig. 1](#);

Figure 1. Regenerative abilities of the newt, *Notophthalmus viridescens*. The eastern red-spotted newt, can regenerate many different organs and tissues, including the upper and lower jaw, eye structures (*e.g.*, the lens, retina, and optic nerve), portions of the brain, the spinal cord, heart, liver, small intestine, and the entire tail and limbs (as can be seen regenerating in the picture). The newt is the only vertebrate with such extensive regenerative abilities maintained as an adult.



Odelberg, 2005). In all tissues in the newt, regeneration is accomplished through the process of epimorphic regeneration and occurs without the formation of a scar, producing a regenerate which is functionally and morphologically indistinguishable from the original.

Many of the vertebrate species listed above are well established laboratory models in developmental biology and have been used to learn much of what is already known about regeneration. Zebrafish and *Xenopus*, for example, have large, transparent embryos, rapid generation times with many offspring, they are relatively amenable to genetic manipulation with well documented genomes, and the animals are large enough to allow easy physical manipulation, while being small enough to house in large numbers. This makes these species ideal laboratory models for studying developmental processes, like regeneration. Axolotls (*Ambystoma mexicanum*), a salamander species with regenerative abilities similar to the newt, are also well developed as a laboratory model. Similar to frogs and fish, they can be bred within a relatively short time, housed in large numbers, and transgenic animals are available. Although less is known about the axolotl genome than frogs and fish, an expressed sequence tag (EST) database is available to researchers. Newts, on the other hand, do not conform to the ideal laboratory model system. They have a very long generation time (approximately five years or longer before reaching sexual maturity) and complex life cycle, they are hard to breed in captivity, they have limited cell lines available (and those that are available are notoriously hard to transfect), and they lack a fully sequenced and annotated genome from which to develop tools for molecular study. Although strides have been made to rectify these issues (Abdullayev *et al.*, 2013; Bruckskotten *et al.*, 2012; Cameron *et al.*,

2004; Casco-Robles *et al.*, 2010; Kumar *et al.*, 2000; Looso *et al.*, 2013; Mercer *et al.*, 2012; Ueda *et al.*, 2005), many of these problems persist.

Eventually, the question is always raised: why not just study regeneration in one of the other vertebrate model systems? First, a zebrafish fin is a vastly different structure than a tetrapod limb; therefore, studying fin regeneration may be more limiting in what we can learn and apply to human medicine. Second, the fact that frogs can only regenerate during the larval stage means that they lose this ability as adults (similar to how humans lose the ability to regenerate digit tips after childhood); thus, if we want to learn more about how to promote regeneration in adults, the frog is not the ideal organism to study. Third, axolotls are neotenic animals, meaning they maintain larval structures as adults (*e.g.*, they maintain gills at sexual maturity), which brings into question whether or not regeneration is also a larval characteristic carried into maturity in this species. Again, the same problem persists here as with the frog: if we want to learn about adult regeneration, the axolotl may not be ideal. This is not to say that these species don't have their uses; certainly, they have helped to unveil many of the processes and common signalling pathways involved in regeneration. But to answer the question posed above, if we want to learn how to improve regeneration in adult humans, we should study a vertebrate organism that can regenerate as an adult. Therefore, despite the drawbacks of the newt as a model laboratory system, researchers continue to study these fascinating creatures because they stand alone among adult vertebrates in their regenerative prowess; that is to say, no other vertebrate, as an adult, is able to perfectly regenerate the number of structures, tissues, and organs that the newt can.

1.3 – Limb Regeneration in the Newt

The gross morphological process of limb regeneration in the newt has been well characterized (Iten and Bryant, 1973). Following amputation, regeneration begins with the formation of a wound epithelium. Beginning just hours after amputation, this single layer of cells grows rapidly to cover the wound and is complete within approximately 24 hours (Alvarado and Tsonis, 2006; Iten and Bryant, 1973; Nye *et al.*, 2003). The wound epithelium is vital to regeneration as it is thought to provide signals to the underlying tissue that promote dedifferentiation and/or cell proliferation (Alvarado and Tsonis, 2006; Whited and Tabin, 2009). At around three to five days post-amputation, the process of dedifferentiation begins. This process involves cells near the site of injury losing their differentiated characteristics and reverting to a progenitor-like state (Chaar and Tsilfidis, 2006; Iten and Bryant, 1973). Dedifferentiation is the key to the newt's regenerative ability and it is characterized by general histolysis of limb tissues, downregulation of differentiation markers, cell cycle re-entry, and upregulation of genes involved in degradation and remodeling of the extracellular matrix (Chaar and Tsilfidis, 2006; Iten and Bryant, 1973; Odelberg, 2005). By about seven days post-amputation, as the dedifferentiated cells proliferate, they begin to form a mass at the regenerating tip, called the blastema. The blastema is the structure which provides the majority of the cells for the newly forming tissue (Iten and Bryant, 1973; Odelberg, 2005).

The presence of nerves has been shown to be a requirement for the formation of the blastema and for regeneration to occur properly *in vivo* (Brockes, 1987). For example, if a limb is denervated before amputation, wound healing and formation of the blastema occur normally (at least morphologically), but the blastema cells fail to

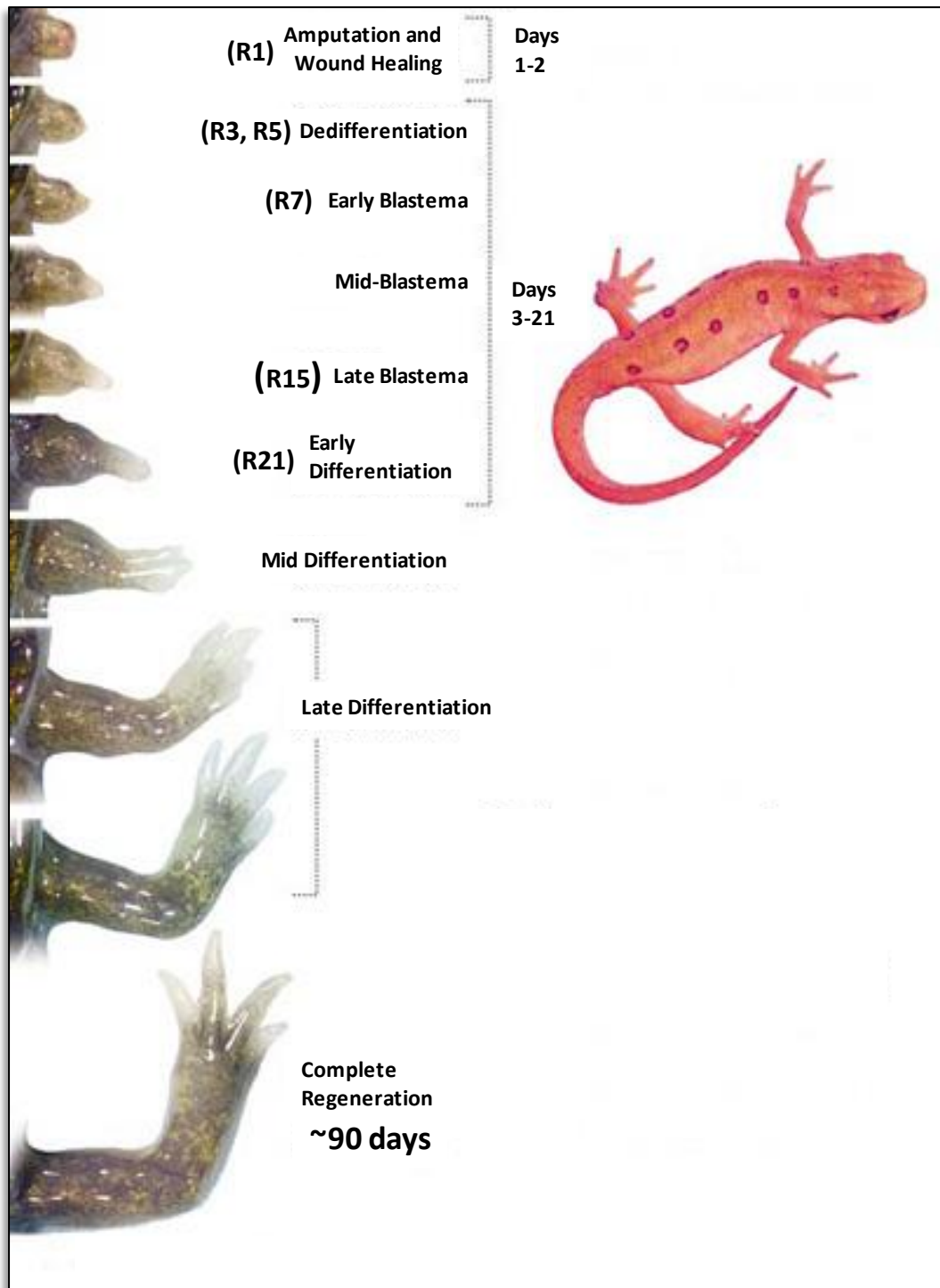
proliferate sufficiently and regeneration is halted (Whited and Tabin, 2009). It is interesting to note that if a limb is manipulated so that it initially develops without a nerve, the limb will regenerate normally following amputation, suggesting that a regenerating limb only requires nerve signals if they were present during embryological development (Whited and Tabin, 2009). One of these nerve signals has been identified: a member of the Anterior Gradient protein family, nAG (newt Anterior Gradient). This protein has been shown to be expressed in the regenerating nerve and the wound epidermis following amputation (Kumar *et al.*, 2007). Newt AG is a secreted ligand which binds to Prod1, a cell surface protein which is expressed in a proximal>distal gradient in the limb (Kumar *et al.*, 2007; Whited and Tabin, 2009). Denervation prevents the expression of nAG in the regenerating limb and overexpression of nAG in a denervated, regenerating limb can rescue the regenerate (Kumar *et al.*, 2007; Whited and Tabin, 2009).

At three weeks post-amputation, the process of redifferentiation begins (Iten and Bryant, 1973). At this stage, cells begin to exit the blastema and differentiate into mature tissues, once again. The patterning of the regenerating limb is thought to recapitulate that which occurs during embryonic limb development, although some minor differences have been identified (Vlaskalin *et al.*, 2004). In a healthy animal, a new limb will regenerate within three months and be functionally and morphologically indistinguishable from the original ([Fig. 2](#)).

1.3.1 – Dedifferentiation: A Key to Regenerative Ability

Similar to what is seen *in vivo*, cultured newt cells have also been shown to exhibit aspects of dedifferentiation. For example, B1H1 (newt blastema cells) and A1

Figure 2. Stages of newt limb regeneration. Immediately following amputation, the process of wound healing begins with the formation of a wound epithelium (R1). Dedifferentiation begins around three to five days post-amputation (R3, R5) and by one week, the early blastema has formed (R7). Approximately 15 days post-amputation, the blastema is fully formed (R15) and, by three weeks, cells have begun to leave the blastema and redifferentiate into limb tissues (R21). In a healthy animal, the entire limb regenerates in about three months and is functionally and morphologically indistinguishable from the original. (Figure adapted from <http://palscience.com/science/can-we-regenerate-our-body-parts-science-say-yes-and-we-are-close/>).

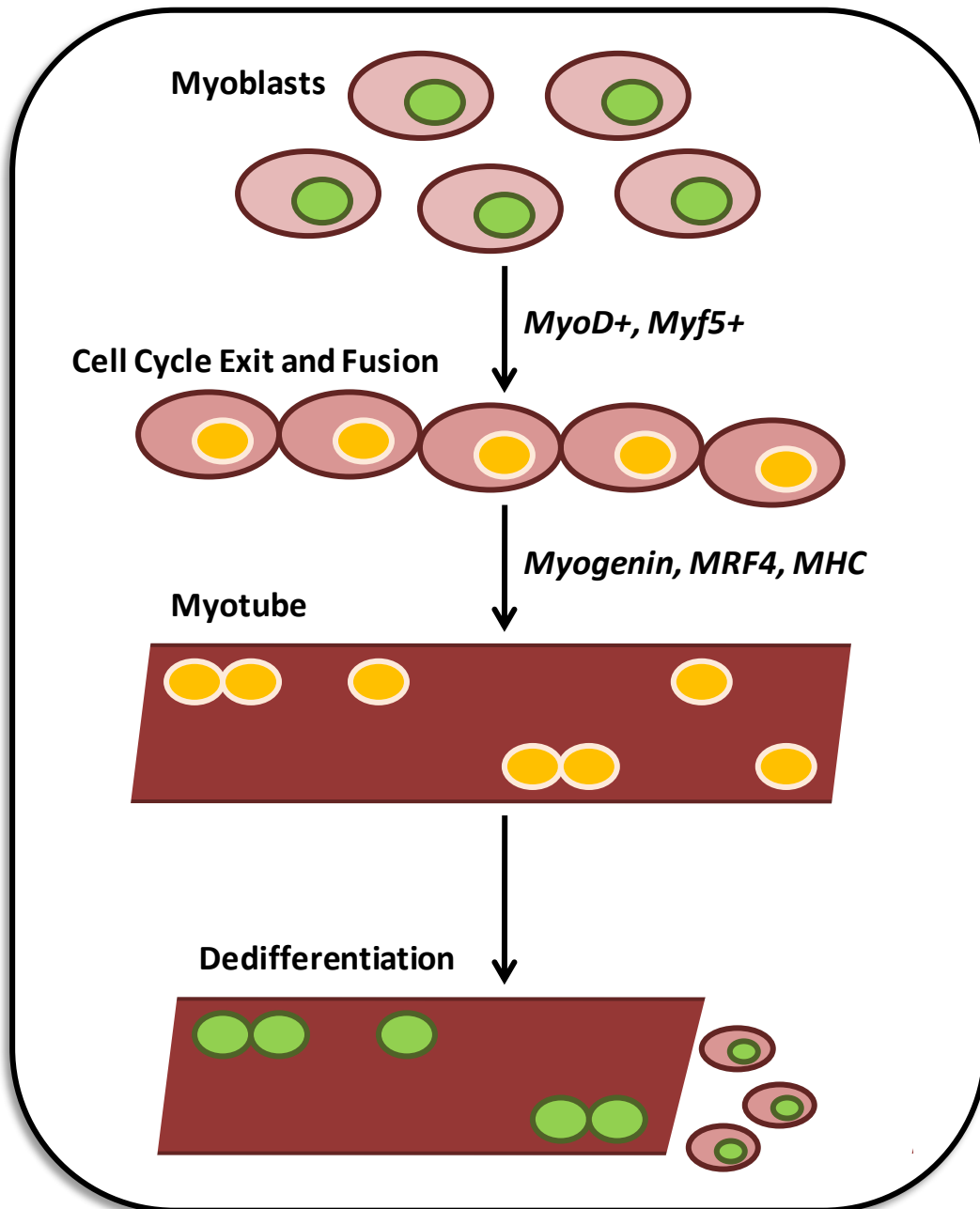


cells (newt myoblasts) can be induced to differentiate into multinucleated myotubes in culture by lowering the serum level ([Fig. 3](#); Beug *et al.*, 2006; Ferretti and Brockes, 1988; Velloso *et al.*, 2001). When the serum level is raised following differentiation, myotube nuclei re-enter the cell cycle, progress through S phase, and arrest in G2 (Beug *et al.*, 2006; Tanaka *et al.*, 1999; Velloso *et al.*, 2001). McGann *et al.* (2001) have also suggested that A1 myotubes can be induced to re-enter the cell cycle following treatment with a protein extract made from regenerating newt limbs and that the myotubes even exhibit cleavage into smaller myotubes and proliferating, mononucleated cells (both of which are key aspects of dedifferentiation; [Fig. 3](#)). The well-defined myoblast differentiation program (Sabourin and Rudnicki, 2000) and the ability of newt A1 myotubes to undergo partial dedifferentiation has made these muscle cells an ideal *in vitro* system in which to study the process of dedifferentiation.

Much research has gone into trying to identify the cellular and molecular triggers for dedifferentiation (both *in vitro* and *in vivo*) and the formation of the blastema in the newt. Tanaka *et al.* (1997) showed that, *in vitro*, newt myotubes require the phosphorylation of retinoblastoma (Rb) protein in order for cell cycle re-entry to occur. Rb is inactivated upon phosphorylation, which causes the release of transcription factors of the E2F family which then translocate to the nucleus and upregulate genes involved in cell cycle initiation (Tanaka *et al.*, 1997). In a follow-up study, they showed that this process was initiated by an as yet unknown factor found in all tested animal sera, which is cleaved and activated by the protease thrombin (Tanaka *et al.*, 1999).

In vivo work has also been carried out to show that newt myotubes can dedifferentiate and contribute to the formation of the blastema. Velloso *et al.* (2000)

Figure 3. Simplified overview of myogenesis and dedifferentiation. Proliferating myoblasts (shown with green nuclei) express myogenic regulatory factors (MRFs) such as *myoD* and *myf5*. When induced to differentiate, myoblasts exit the cell cycle (shown with yellow nuclei) and begin to fuse together, forming a syncitium. Multinucleated myotubes are formed with the expression of *myogenin*, *MRF4*, and *myosin heavy chain (MHC)*. In newt myotubes, exposure to high serum or thrombin-activated serum factor causes cell cycle re-entry of the myotube nuclei (green). If exposed to a protein extract from regenerating newt limbs, myotube nuclei will re-enter the cell cycle and proliferating, mononucleated cells will bud off.



showed that post-mitotic myotubes implanted into regenerating limbs can break down into mononucleated cells and contribute to the formation of the blastema (even in the absence of cellular proliferation). In the same year, Kumar *et al.* showed that myotubes implanted in a regenerating limb could not only break down into mononucleated cells to aid in blastema formation, but these mononucleated cells were then able to proliferate (Kumar *et al.*, 2000).

There has been much debate in the literature over the dedifferentiation of cells, mostly surrounding the question of whether cells revert to an embryonic-like pluripotency or if they only go so far as tissue-specific progenitors. Originally, histological evidence indicated the blastema was composed entirely of a homogeneous mass of undifferentiated progenitor cells derived from all tissues of the limb stump (Kragl *et al.*, 2009). Recently, Kragl *et al.* (2009) shed some light on this issue by tracking major limb tissues during regeneration, which were transplanted from transgenic axolotls (ubiquitously expressing green fluorescent protein, GFP) into wild-type axolotls. Their data indicated that, at least in this species, the blastema is composed of a heterogeneous mixture of restricted progenitor cells, with only dermis being able to give rise to multiple cell types (Kragl *et al.*, 2009). Even more recently, Morrison *et al.* (2010) showed that muscle satellite cells in the newt are able to contribute to the blastema and give rise to tissues other than just muscle. This data set, in contrast to the work of Kragl *et al.* (2009), implies the blastema is not simply a pool of restricted progenitors.

Similar to the debate over the extent of the dedifferentiation of cells, it has been debated whether the regenerating limb is derived entirely from dedifferentiated cells or if other adult stem cell populations [*i.e.*, muscle satellite cells, side population (SP) cells,

haematopoietic stem cells, etc.] play a significant role (Bryant *et al.*, 2002; Morrison *et al.*, 2010). Although it is known that adult stem cells do contribute to the regenerating limb (Morrison *et al.*, 2006; Morrison *et al.*, 2010) a precise quantification of the relative contribution of stem cells versus dedifferentiated cells has yet to be determined.

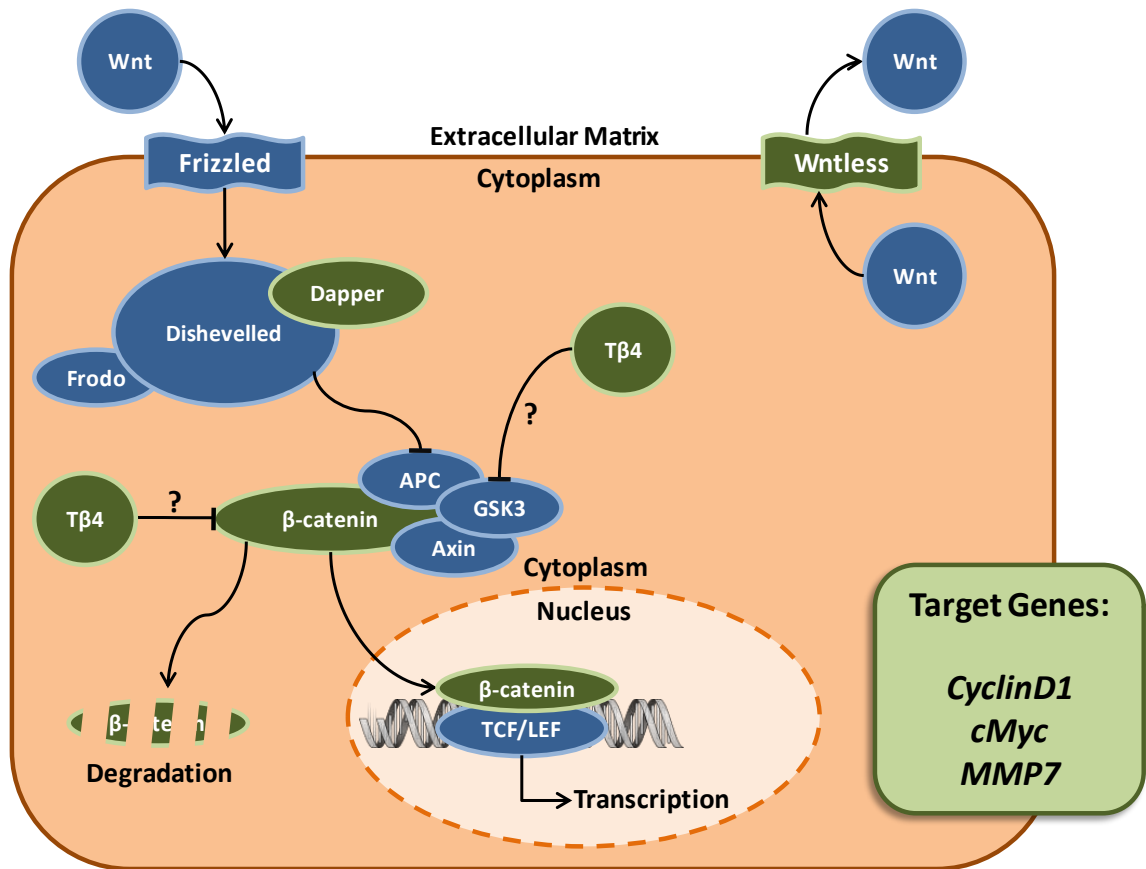
1.3.2 – Molecular Aspects of Dedifferentiation and Regeneration

One gene that has been implicated in the regeneration response in newts is *msx1*, a homeobox gene encoding a transcriptional repressor that has been associated with bone morphogenetic protein (BMP) signalling (Crews *et al.*, 1995; Odelberg, 2004; Odelberg, 2005). *Msx1* has been shown to inhibit differentiation of several mesenchymal and epithelial progenitor cell types, to be involved in microtubule disassembly during salamander myotube cellularization, and to cause cellularization of multinucleated, mouse myotubes into proliferating, mononucleated cells (in combination with serum stimulation; Crews *et al.*, 1995; Echeverri and Tanaka, 2002; Kumar *et al.*, 2004; Odelberg, 2004). Interestingly, clonally derived populations of these mononucleated cells were shown to transdifferentiate into cells expressing markers for cartilage, fat, and bone (Odelberg, 2004). This indicates that *msx1* may not only induce break down of myotubes, but have some reprogramming effects as well. Although Barker and Beck (2009) showed that *msx1* overexpression could not rescue regeneration in refractory stage *Xenopus* hindlimbs, it has been shown to rescue regeneration in *Xenopus* tails and mouse digit tips upon overexpression of *msx1*, itself, or downstream BMP targets (Odelberg, 2005). These data indicate that *msx1* and the BMP pathway are important factors for regeneration in multiple species.

Matrix metalloproteinases (MMPs) are a family of proteases which can degrade nearly all proteins found in the extracellular matrix (ECM; Odelberg, 2005). The activity of these molecules is also known to be increased during the early stages of limb regeneration in the newt (Odelberg, 2005). Vinarsky *et al.* (2005) investigated the activity of three MMPs specific to the newt (*MMP3/10a*, *MMP3/10b*, and *MMP9*) and found that they were all upregulated within hours following limb amputation. Furthermore, inhibition of MMP activity produced either a malformed limb regenerate or a stump exhibiting a scar (Vinarsky *et al.*, 2005). Related to this, *tissue inhibitor of metalloproteinase 1* (*TIMP1*; an inhibitor of MMP proteolytic activity) was shown by Stevenson *et al.* (2006) to be upregulated during wound healing and dedifferentiation in regenerating limbs. This suggests that MMPs are tightly regulated during the early stages of regeneration and provides further evidence of a role for this protein family in regeneration.

One might hypothesize that, with regeneration being so widespread throughout the animal kingdom, there should be an evolutionary basis for this phenomenon. That is to say, there must be a number of hallmarks of regeneration which are shared among animals possessing this ability to one extent or another. Indeed, in recent years, some of these hallmarks have come to light. One such example, the wnt signalling pathway ([Fig. 4](#)), has been studied in some detail among different species with various regenerative abilities. Wntless, for example, which is a transmembrane protein involved in the secretion of wnt proteins for propagation of cell-cell signalling (Bänziger *et al.*, 2006), has been shown by Adell *et al.* (2009) to be required for regeneration in planarians. For example, RNAi experiments on the planarian *wntless* homologue (*smed-evi*) caused two-

Figure 4. Simplified overview of the wnt signalling pathway. Wnt ligands bind to the frizzled receptor and activate the dishevelled complex. The dishevelled complex binds and inhibits the APC/GSK3/axin complex, which normally binds cytosolic β -catenin and targets it for degradation. Upon removal of the APC/GSK3/axin complex, cytosolic β -catenin accumulates and translocates to the nucleus where it combines with transcription factors of the TCF/LEF family to induce transcription of downstream target genes (*e.g.*, *cyclinD1*, *cMyc*, *MMP7*, etc.). When abundant dapper protein is present, it antagonizes the dishevelled complex, leading to an increase in the amount of the APC/GSK3/axin complex and, therefore, a decrease in the amount of cytosolic β -catenin available (Cheyette *et al.*, 2002). Wntless is a G-protein coupled receptor that is important for secreting wnt proteins from signalling cells (Bänziger *et al.*, 2006). Reports suggest that thymosin- β 4 (T β 4) may be a negative regulator of GSK3 and β -catenin (Ryu *et al.*, 2012).



headed regenerates and defects in the regeneration of the nervous system (Adell *et al.*, 2009). Wnt signalling through β -catenin, a transcription factor involved in regulating many different cellular processes, has also been shown to be key, not only for the initiation of the regeneration response, but also for promoting the process in species as diverse as zebrafish, frogs, and axolotls (Kawakami *et al.*, 2006; Yokoyama *et al.*, 2007). For example, overexpression of antagonists of β -catenin and wnt signalling [axin and dickopf (dkk), respectively] in regenerating limbs of axolotl and *Xenopus* inhibited regeneration and the formation of distal limb elements (Kawakami *et al.*, 2006). Dkk overexpression in zebrafish tails also inhibited fin regeneration (Kawakami *et al.*, 2006). In some cases, overexpression of a constitutively active form of β -catenin was able to partially or completely rescue limb/fin regeneration (Kawakami *et al.*, 2006). Yokoyama *et al.* (2007) showed that overexpressing dkk immediately prior to amputation or during early blastema formation blocked limb regeneration in *Xenopus* tadpoles. The wnt/ β -catenin pathway has even been implicated in the reprogramming of aged, human epidermal cells (Zhang *et al.*, 2012b), a process vital to regeneration.

Another gene that has been implicated in regeneration is *thymosin- β 4* (*T β 4*). The T β 4 peptide is a ubiquitously expressed, actin-sequestering molecule with many other biological activities, including downregulating inflammatory chemokines/cytokines, promotion of cell migration, blood vessel formation, cell survival, and stem cell maturation (Goldstein *et al.*, 2012; Philp and Kleinman, 2010). T β 4 has been shown to enhance wound repair in mammals by preventing scar formation, apoptosis, inflammation, and bacterial growth (Ehrlich and Hazard, 2010; Goldstein *et al.*, 2012). In injured mouse skeletal muscle, T β 4 and a related peptide, T β 10, were both upregulated

in muscle fibers and inflammatory haematopoietic cells during the early stages of regeneration (Tokura *et al.*, 2011). T β 4 has been shown to be chemotactic for endothelial cells, keratinocytes, and cardiomyocytes, while being inhibitory for the chemotaxis of neutrophils (Tokura *et al.*, 2011). T β 4 has also been shown to enhance survival and repair of cardiomyocytes following cardiac injury via signalling through Akt kinase (Tokura *et al.*, 2011). T β 4 has even been shown to initiate the embryonic development program in adult mouse heart muscle (Bock-Marquette *et al.*, 2009). Despite the recent interest in the role of T β 4 in wound healing and regeneration, relatively little is known about the extracellular pathways through which T β 4 signals. Interestingly, T β 4 has also been implicated as a potential regulator of wnt signalling (Ryu *et al.*, 2012; [Fig. 4](#)), providing further evidence it may be linked to other pathways involved in regeneration.

1.4 – Potential for Mammalian Muscle Dedifferentiation

For most vertebrates, including reptiles, birds, and mammals, the normal response to injury as an adult is wound healing and scar formation (Yokoyama, 2008). However, mammalian muscle does possess a limited capacity for regeneration following injury, albeit using a different process than that observed in newts. In mammals, muscle regeneration requires populations of multipotent adult stem cells residing in the muscle tissue [satellite cells and muscle side population (SP) cells] which differentiate into myoblasts, among other cell types, following injury (Gussoni *et al.*, 1999; Morgan and Partridge, 2003; Sabourin and Rudnicki, 2000; Uezumi *et al.*, 2006). The myoblasts then fuse with each other and mature into myofibers, thus repairing the muscle (Chargé and Rudnicki, 2004; Sabourin and Rudnicki, 2000). Without these stem cells, mammalian muscle is normally unable to repair and regenerate following injury.

At first glance, the fact that mammalian myotubes appear to be refractory to cell cycle re-entry (and thus, dedifferentiation) may indicate that the ability to dedifferentiate has been lost in mammalian cells. However, research has shown that the same thrombin-activated serum factor described above (which causes cell cycle re-entry in newt myotubes; see [Section 1.3.1](#)) can induce a response from immediate early genes involved in cell cycle re-entry in mouse C2C12 myotubes (Löf *et al.*, 2007). Also, when mouse C2C12 myoblasts were co-cultured with newt A1 myoblasts, and differentiation was induced to form newt-mouse hybrid myotubes, high-serum stimulation not only induced cell cycle re-entry in newt nuclei within the myotube, but also the mouse nuclei (Velloso *et al.*, 2001). This indicated that the newt nuclei (which were responding to the thrombin-activated serum factor through Rb phosphorylation) were able to produce a factor (or factors) which could induce a cell cycle re-entry response in the mouse nuclei within the same myotube. Furthermore, McGann *et al.* (2001) showed that a protein extract made from regenerating newt limbs (which induced dedifferentiation in newt myotubes; see [Section 1.3.1](#)) could also induce cell cycle re-entry and breakdown of mammalian myotubes into proliferating, mononucleated cells. Another study showed that inactivation of Rb (in combination with another protein, alternative reading frame, ARF) in mouse primary myotubes caused cell cycle re-entry (Pajcini *et al.*, 2010). Together these data indicate that, while mammalian myotubes on their own do not exhibit a dedifferentiation response, they can be induced to both re-enter the cell cycle and break down into mononucleated cells when supplied with the appropriate signals.

Beyond the *in vitro* studies described above, a mouse strain (Murphy Roths Large, MRL) which lacks the cyclin-dependent kinase inhibitor, p21, has been shown to exhibit

healing and regeneration properties somewhat similar to those of newts (Arthur and Heber-Katz, 2011; Bedelbaeva *et al.*, 2010). MRL mice can completely close ear hole puncture wounds through formation of a circular blastema, they can regrow articular cartilage, and partially regenerate amputated digits (Bedelbaeva *et al.*, 2010). These studies indicate that mammals are capable of more extensive *in vivo* regeneration, beyond simply *in vitro* cell cycle re-entry and breakdown of myotubes.

1.5 – Objectives and Experimental Approach

The cellular and molecular mechanisms of limb development are highly conserved among tetrapods (Galis *et al.*, 2003; Yokoyama, 2008). This leads to the question of why the normal response to limb amputation, for example, is so different between urodele amphibians and mammals? Yokoyama (2008) suggests that it is the molecular events early on in regeneration which determine the extent of the response to injury; that is, the early signals determine whether regeneration will consist simply of wound healing and scarring or the re-development of a complete limb.

Despite the fact that the regenerative abilities of the newt have been known for almost 250 years (Gargioli and Slack, 2004; Odelberg, 2005; Whited and Tabin, 2009), we still know very little about the cellular and molecular processes underlying this phenomenon. The main factor contributing to this paucity of knowledge is the lack of genome information and, consequently, a lack of genetic tools. Salamander genomes range from approximately 10pg to 135pg (average = 35pg), meaning there is about ten times the genetic material in the average salamander genome compared to the human genome (a likely explanation for why the newt genome project has not been undertaken!). Much of the past research on newt regeneration has focused on studying the process

separately in various organs and structures (*e.g.*, cardiac muscle, spinal cord, limb, jaw, lens, and retina; Borchardt and Braun, 2007; Echeverri and Tanaka, 2003; Kurosaka *et al.*, 2008; Makarev *et al.*, 2006; Maki *et al.*, 2010a; Maki *et al.*, 2010b). On one hand, this approach has provided researchers with a short list (approximately 400 sequences) of newt-specific gene homologues, microRNAs, and proteins, as well as information about how these various genes and other factors may play a role in the regeneration process. Unfortunately, with such a specific and directed approach, this research has only been able to slowly chip away at the larger picture of the diverse cellular and molecular mechanisms underlying regeneration.

Our main objectives for this work were to identify some of the genes involved in dedifferentiation in the newt and to provide a functional analysis of some of these genes throughout regeneration in different tissues. We decided to take a broader approach to identifying candidate genes using representational difference analysis (RDA). This technique compares two populations of mRNA, subtracts the sequences shared between the two populations, and then amplifies the remaining sequences which are unique to each population. The main benefit of RDA is that it provides an unbiased approach to identifying a gene pool representing the experimental condition (in this case, the distal regenerating tip of a limb during dedifferentiation). We also decided to use microarrays as a tool to both verify the RDA and perform further expression analysis of our candidate genes by comparing them to genes from different time points of regeneration in both limb and tail. Knowing the expression of candidate genes at various time points of limb regeneration would allow us to begin identifying specific genes for follow-up study by choosing those with interesting profiles (*e.g.*, a gene which is upregulated early on and

downregulated following blastema formation may indicate an important role in dedifferentiation). Furthermore, comparing the limb profiles to a profile of candidate genes during tail regeneration would allow us to choose genes which have a common profile in more than one regenerating tissue, thus providing stronger evidence for a common role in regeneration.

For functional analysis, we decided to overexpress candidate genes in newt and mouse muscle cells. These cells provide an ideal system in which to test candidate genes for disruption of the differentiation process or induction of cell cycle re-entry or myotube breakdown (all of which indicate a potential role in dedifferentiation).

This work will provide a large number of novel newt genes to add to the growing list and provide further information about the newt transcriptome. Importantly, our approach will specifically identify genes relevant to the dedifferentiation and regeneration process. In taking the broad approach we have outlined, we may also be able to begin identifying gene pathways and interactions between gene pathways that may play a role in dedifferentiation and regeneration. This will allow us to begin looking at the regeneration process as a whole rather than dissecting out individual genes upon which to focus.

A better understanding of the early dedifferentiation signals and their interactions in the newt may help elucidate new ways of enhancing regeneration in mammals. This work could also have an impact on human regenerative medicine through the possible development of new treatments and potential cures for human health issues ranging from degenerative diseases, to spinal cord injuries, myocardial infarction, developmental pathologies, ageing, cancer, and even limb amputations.

MATERIALS AND METHODS

2.1 – Animal Housing

Newts (*Notophthalmus viridescens*) were supplied by Charles D. Sullivan Co., Inc. (USA) and maintained on a 12 hour light/dark cycle. Newts were housed at 22°C in large plastic containers with constant aeration and water flow and were fed live blackworms (*Lumbriculus variegates*) twice weekly. Animal care and use was approved by the University of Ottawa Animal Care and Veterinary Service Committee.

2.2 – Collection of Regenerating Tissue

Newts were anaesthetized in 0.05% MS-222 (see [Appendix III](#)) and initial amputations were made at the level of the wrist (for forelimb regenerates) or approximately 1.5cm from the distal end of the tail (for tail regenerates). The autopod was collected as intact limb tissue (R0) and an approximately 1mm piece of tissue was collected from the distal end of the freshly amputated tail for intact tail tissue (T0). These tissues were flash frozen in liquid nitrogen and stored at -80°C until RNA was isolated. Newts were allowed to recover in dechlorinated tap water before being transferred back to housing tanks. Three days after the initial amputation, the same protocol was followed with only the distal 1mm of regenerating tissue [limb (R3) or tail (T3)] being amputated and frozen. In general, tissue from at least three newts was combined to account for biological variation.

For long term regeneration profile experiments, regenerating tissue was collected at multiple time points representing various stages of limb/tail regeneration ([Fig. 2](#)) from

initial wound healing (R1), through peak dedifferentiation (R3/T3, R5), early blastema (R7/T7), late blastema (R15/T15) and the beginning of redifferentiation (R21).

2.3 – RNA Isolation and Concentration

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, USA) according to the manufacturer's instructions. Briefly, tissue was ground and lysed using a microcentrifuge pestle in lysis buffer (or cell pellets were lysed by vortexing in lysis buffer). Samples were loaded into RNeasy Spin Columns, washed, and RNA was eluted in water. Tail RNA to be used for microarray analysis was concentrated using the NucleoSpin RNA XS Kit (Clontech, USA) according to the manufacturer's instructions. In short, RNA samples were combined, mixed with lysis-binding buffer, and loaded onto NucleoSpin RNA XS Columns. RNA was washed and eluted in water and concentration was determined on a Beckman Coulter DU-800 Spectrophotometer (Beckman Coulter, USA) or a Nanodrop 2000 (Thermo Scientific, USA). RNA quality was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, USA; performed by StemCore Laboratories, Canada) or by running on a 1.5% agarose gel for 15 minutes and visualizing on a Gel Doc XR+ System (Bio-Rad, USA). All RNA samples were stored at -80°C.

2.4 – Representational Difference Analysis (RDA)

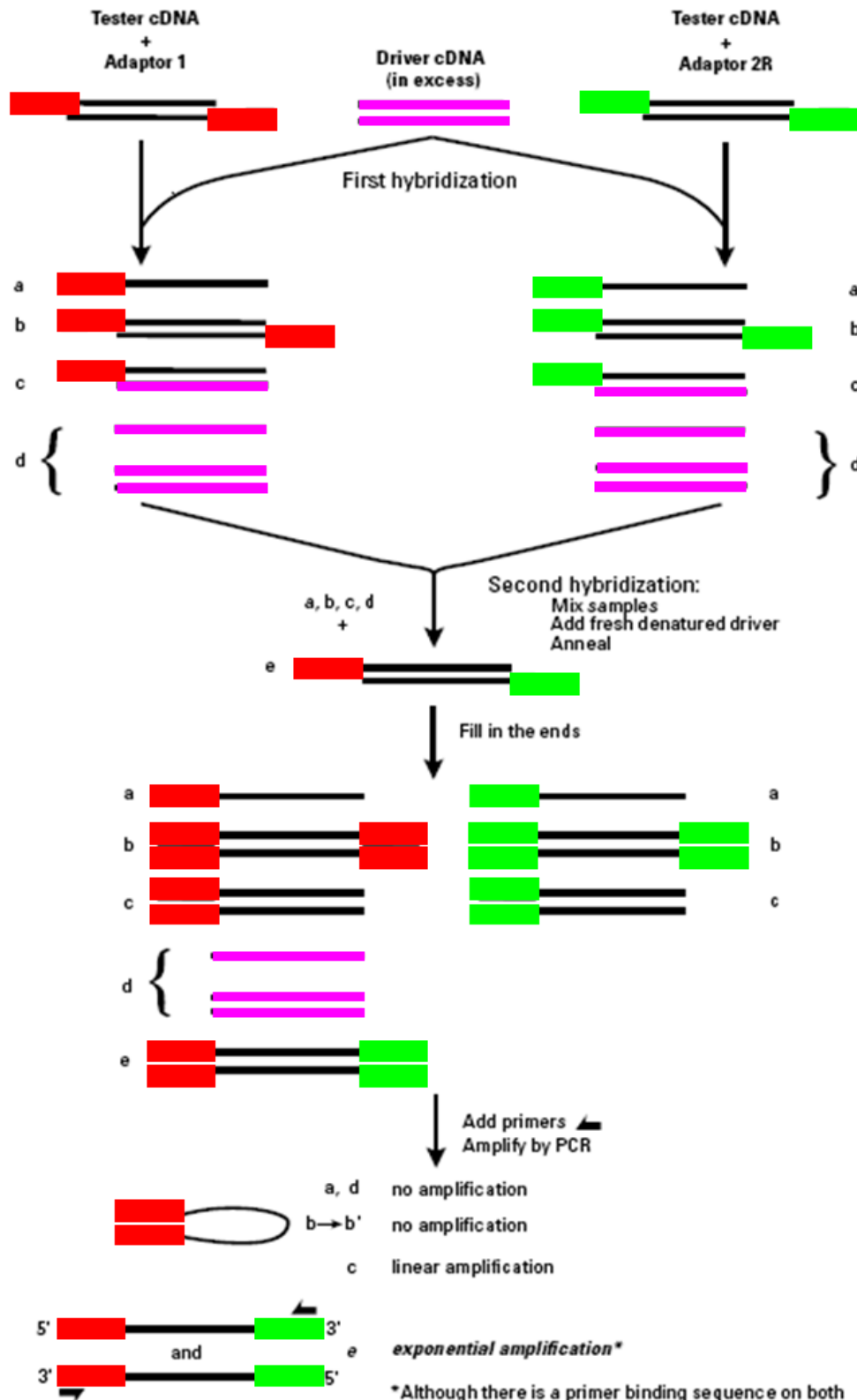
Complementary DNA was prepared using the SMART PCR cDNA Synthesis Kit (Clontech, USA) following the manufacturer's instructions. In short, 1µg each of R0 and R3 RNA was used for the first strand cDNA synthesis with SuperScript III Reverse Transcriptase (Invitrogen, USA). Following second strand synthesis, the cDNA samples

were amplified through 17 PCR cycles and the products were digested with *Rsa*I. Digested cDNA was purified using the MinElute Reaction Cleanup Kit (Qiagen, USA) according to the manufacturer's instructions. The RDA was then carried out with the PCR-Select cDNA Subtraction Kit (Clontech, USA) following the manufacturer's instructions ([Fig. 5](#)). Briefly, tester (R0) cDNA was split into two pools and each was ligated to a unique adaptor. Each adaptor-ligated tester pool was then subjected to a first hybridization with driver (R3) cDNA. Then the two tester/driver hybrid pools were combined and hybridized a second time with fresh driver and subjected to PCR amplification. This process was repeated after switching the tester and driver to achieve a reverse subtraction. The product of the RDA was two pools of cDNA: one pool enriched for mRNA expressed in the intact limb (*i.e.*, genes downregulated during dedifferentiation) and one pool enriched for mRNA expressed in the three day regenerate (*i.e.*, genes upregulated during dedifferentiation).

2.5 – Cloning and Identification of cDNAs

Complementary DNA from the two RDA pools was cloned into one of two vectors: the pCR4-TOPO vector (TOPO TA Cloning Kit, Invitrogen, USA) or the pGEM-T Easy vector (Promega, USA). TOPO vector was transformed into *Escherichia coli* TOP10 cells (Invitrogen, USA) or *E. coli* JM109 cells (Promega, USA) while pGEM-T Easy vector was transformed into *E. coli* JM109 cells only. Briefly, approximately 50ng of each cDNA pool was mixed (separately) with competent *E. coli* and incubated on ice for 30 minutes. Cells were heat shocked at 42°C for exactly 30 seconds and returned immediately to ice. The transformed cells were incubated in SOC media (New England BioLabs, USA) for one hour at 37°C to recover and were plated on

Figure 5. Overview of the RDA procedure. RDA was performed with the PCR-Select cDNA Subtraction Kit (Clontech, USA). In short, one cDNA sample (tester; *e.g.*, R0) was split into two and each pool was ligated to a unique adaptor (red and green blocks). Each adaptor-ligated tester pool was then subjected to a first hybridization with driver (*e.g.*, R3) cDNA. Next, the two tester/driver hybrid pools were combined and hybridized a second time with fresh driver (resulting in formation of “e” type molecules) and subjected to PCR amplification (to exponentially amplify “e” type molecules). This step will only amplify the cDNAs which are unique to the tester population (*i.e.*, “e” type molecules) as cDNAs common to both tester and driver groups will hybridize to each other and lack the appropriate linkers for exponential amplification. The process was repeated after switching the tester and driver to achieve a reverse subtraction. The final product of the RDA was two pools of cDNA: one pool enriched for mRNA expressed in the intact limb (*i.e.*, genes downregulated during dedifferentiation) and one pool enriched for mRNA expressed in the three day regenerate (*i.e.*, genes upregulated during dedifferentiation). (Figure adapted from PCR-Select cDNA Subtraction Kit User Manual, Clontech, USA.)



*Although there is a primer binding sequence on both ends of the type *e* molecules, the shorter overall homology at the two ends effectively negates the suppression PCR effect—except for very short molecules. See Appendix A for more details on suppression PCR.

LB agar plates (see [Appendix III](#)) containing 50µg/ml Ampicillin (Sigma, USA). In the case of pGEM-T Easy vector, which allows for blue/white screening, IPTG (0.1mM final concentration; Invitrogen, USA) and X-gal (40µg/ml final concentration; Invitrogen, USA) were spread on the agar prior to plating the transformed bacteria.

To make glycerol stocks, colonies were picked from the initial plates and grown overnight at 37°C in 96-well culture plates containing LB media (see [Appendix III](#)). Glycerol stocks were made by combining 700µl of cell culture with 300µl of sterile, 50% glycerol (Fisher, USA) and freezing at -80°C. Scrapings were taken from the glycerol stocks and colony PCR (see [Appendix I](#)) was performed with the GoTaq Green PCR Master Mix (Promega, USA) and vector specific primers (M13 Forward/Reverse, see [Appendix II](#)) to determine the insert size. PCR reactions were cleaned using microCLEAN (Gel Company, USA), following the manufacturer's instructions. Briefly, an equal volume of microCLEAN reagent was added to the PCR reaction and incubated at room temperature for five minutes. The mix was centrifuged for 50 minutes at 2550xg in a Sorvall Legend Micro 21 R benchtop centrifuge (Thermo Scientific, USA) and the DNA pellet was resuspended in water. Sequencing was performed by Operon (USA) and clones were identified using the basic local alignment search tool (BLAST; <http://blast.ncbi.nlm.nih.gov/>).

2.6 – Microarray

Cloned inserts from the RDA were amplified with vector specific primers (T7 Promoter, T3, or SP6; see [Appendix II](#)) and cleaned with microCLEAN as above (see [Section 2.5](#)), except the DNA pellets were resuspended in 50% DMSO (Invitrogen, USA). These were spotted onto slides using the SpotArray (Perkin Elmer, USA). The

arrays were printed with the clones identified in the RDA, other known newt cDNAs (*i.e.*, published sequences), and other cDNAs identified from previous work in our lab.

Microarrays were probed with cDNA obtained from regenerating limbs and tails at various time points throughout regeneration (post-amputation days 1, 3, 5, 7, 15, and 21 for limb and days 3, 7, and 15 for tail). RNA was isolated as above (see [Section 2.3](#)) and converted to cDNA using the 3DNA Array 900 Kit (Genisphere, USA) following the manufacturer's instructions. In short, RNA was incubated for two hours at 42°C with the reverse transcription mix and one of two fluorescent dyes (Cy3 or Cy5). Microarray slides were washed before drying by centrifugation. Labelled cDNAs were hybridized to microarrays overnight at 42°C and washed the next day at 55°C. Finally, 3DNA Capture Reagent was bound to the labelled cDNA on the microarray and the arrays were scanned using the ScanArray (Perkin Elmer, USA) and ProScan software (Perkin Elmer, USA). The array data were uploaded to an online tool, Webarray (<http://www.webarraydb.org/webarray/index.html>), for normalization and final analysis (performed by Dr. Alan Mears).

2.7 – Rapid Amplification of cDNA Ends (RACE)

In order to further analyze genes of interest identified in the RDA/microarray, RACE was performed to obtain full length sequences of several genes. Three protocols were used: “classic” 5'/3' RACE, RNA Ligase Mediated (RLM)-RACE, and bubble PCR. For classic 5' RACE, RNA was reverse transcribed using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, USA) following the manufacturer's instructions. In short, 1µl of RNA was denatured at 75°C for five minutes and reverse transcribed with the appropriate gene specific outer primer (see [Appendix II](#)). The

reaction was cleaned using Microcon Ultracel YM-30 columns (Millipore, USA) and cDNA was eluted in 10µl of DNase-free water for polyA-tailing. Terminal deoxynucleotidyl Transferase (TdT; Roche, USA) was used for the terminal transferase reaction at 37°C for 15 minutes. The polyA-tailing reaction was stopped by incubating for three minutes at 80°C and the reaction was diluted to 1ml in 10mM tris/1mM EDTA (TE, pH 7.5; Fisher, USA/Bioshop, Canada). From this cDNA sample, 10µl was used in the RACE PCR protocol (see [Appendix I](#)) with a polyT- and adapter primer (see [Appendix II](#)) and the appropriate gene specific inner primer (see [Appendix II](#)) along with the FidelityTaq 2x PCR Mastermix (Affymetrix, USA). For classic 3' RACE, the same protocol was used except the initial reverse transcription was carried out with a polyA-primer.

In order to achieve more full-length sequences, the FirstChoice RLM-RACE Kit (Ambion, USA) was used according to the manufacturer's instructions to perform RNA Ligase Mediated RACE. For 5' RLM-RACE, mRNA was first treated with calf intestine alkaline phosphatase (CIP) to remove 5' phosphate groups from any degraded mRNA. The mRNA was then treated with tobacco acid pyrophosphatase (TAP) to remove the 5' cap from full-length mRNA. An adapter sequence (with known, internal primer sites) was ligated to the 5' end of the modified mRNA and reverse transcription and PCR were performed. For 3'RLM-RACE, the only modifications to the mRNA were the addition of a polyT-primer/adapter sequence (with known, internal primer sites) to mediate reverse transcription and PCR.

Genes for which we had not been able to achieve full length sequences with the first two RACE methods were subjected to bubble PCR (adapted from Chinen *et al.*,

2008) by Dr. Alan Mears. Genomic DNA was isolated from newt tissue using the DNeasy Blood and Tissue Kit (Qiagen, USA), following the manufacturer's instructions. Briefly, tissue samples were digested in lysis buffer (with proteinase K) at 56°C for approximately three hours. Ethanol (Commercial Alcohols, Canada) was added to precipitate the DNA and the mixture was loaded into a DNeasy Mini spin column. Genomic DNA was washed and eluted in 200µl of elution buffer. The genomic DNA was then digested with five separate, blunt-cutting restriction enzymes: *DraI*, *RsaI*, *HaeIII*, *EcoRV*, and *SmaI* (New England BioLabs, USA). Bubble oligos with internal primer sites (see [Appendix II](#)) were ordered (IDT, USA) and hybridized together to form the bubble adaptor (see [Appendix II](#)). These adaptors were ligated to the digested genomic DNA fragments using T4 DNA ligase (Invitrogen, USA). The ligated bubble adaptor/genomic DNA fragments were then subjected to PCR (see [Appendix I](#)) in which the first strand is made using a gene specific primer (see [Appendix II](#)). This first strand creates the priming site for the outer bubble primer (see [Appendix II](#)). The amplicon from the first set of primers is subjected to PCR with a set of nested primers (gene specific and bubble specific, see [Appendix II](#)) which amplify the final product.

2.8 – Quantitative Polymerase Chain Reaction (qPCR)

RNA was isolated from tissues or cells as described above (see [Section 2.3](#)). Potential contaminating genomic DNA was removed using the DNA-free Kit (Ambion, USA) following the manufacturer's instructions. Briefly, DNase was added to approximately 4µg of total RNA and incubated for 30 minutes at 37°C. DNase inactivation reagent was added and the mixture was centrifuged at maximum speed for one minute in a Sorvall Legend Micro 21 R benchtop centrifuge (Thermo Scientific,

USA). The supernatant (containing RNA) was used to make cDNA with the iScript cDNA Synthesis Kit (BioRad, USA) according to the manufacturer's instructions. In short, 2µg of RNA was used per reaction and the final product was diluted to 200µl with water. The qPCR (see [Appendix I](#)) was carried out with the iQ SYBR Green Supermix (BioRad, USA) on a MyIQ iCycler (BioRad, USA) and the data were analyzed using the MyIQ software (BioRad, USA). All primers used for qPCR are listed in [Appendix II](#).

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was chosen as a normalization gene for all qPCR experiments on newt cells/tissue based on a previous study which compared the expression of newt *GAPDH* and *β-actin* during newt forelimb regeneration (Vascotto *et al.*, 2005). The expression of *GAPDH* was found to be ubiquitous during limb regeneration with no overall variation in expression levels, while *β-actin* expression was found to be heterogeneous (Vascotto *et al.*, 2005). For mouse cells, *GAPDH* and *hypoxanthine-guanine phosphoribosyltransferase (HPRT)* were used as normalization genes.

2.9 – Tissue Sections of Regenerating Limbs

Newts were anaesthetized and their forelimbs were amputated as described above (see [Section 2.2](#)). The regenerating limbs were collected at time points throughout regeneration corresponding to peak dedifferentiation (R3), early blastema (R7), late blastema (R15), and the beginning of redifferentiation (R21). For this experiment, however, the regenerating limbs were reamputated through the mid-humerus. Intact limbs were also collected by amputating through the mid-humerus. Limbs were fixed in 4% paraformaldehyde (PFA; Sigma, USA) overnight at 4°C and washed with phosphate-buffered saline (PBS; see [Appendix III](#)) the following day. Decalcification was carried

out with an EDTA-based decalcification solution (see [Appendix III](#)) over five days at 4°C with daily solution changes. The fixed and decalcified limbs were then cryoprotected with 30% sucrose (EMD, USA) overnight at 4°C. The limbs were equilibrated in a 50:50 mixture of 30% sucrose:Optimal Cutting Temperature compound (OCT; Sakura, USA) overnight at 4°C before being frozen on liquid nitrogen in either the 50:50 mix (for harder embedding medium) or OCT alone (for softer embedding medium). Limbs were stored at -80°C until sectioning.

Longitudinal sections of limbs were cut at a 10µm thickness using a Shandon Cryotome FSE cryostat (Thermo, USA) and adhered to Superfrost Plus microscope slides (Fisher, USA). Additional sectioning was performed by the Department of Pathology and Laboratory Medicine at the University of Ottawa.

2.10 – Haematoxylin and Eosin (H & E) Staining of Regenerating Limb Tissue Sections

Before performing immunohistochemistry (IHC) for specific antibodies, a haematoxylin and eosin (H & E) stain was performed on limb sections to provide a picture of the overall morphology and anatomy of longitudinal limb sections. Briefly, frozen sections were allowed to return to room temperature before being rehydrated in water for three minutes and post-fixed with 4% PFA (Sigma, USA) for another three minutes. The fixative was washed off under running tap water for five minutes. Nuclei in limb sections were stained with filtered haematoxylin (Fisher, USA) for approximately one minute and rinsed in running tap water for three minutes. Slides were dipped three times in 70% ethanol (Commercial Alcohols, Canada) containing five drops of concentrated hydrochloric acid (Fisher, USA) and rinsed with five dips in tap water.

Slides were then dipped ten times in filtered, saturated lithium carbonate (EMD, USA), diluted 1:2 with water, followed by a ten minute wash in tap water. The sections were counterstained in eosin Y (see [Appendix III](#)) for one minute to stain cytoplasm. Slides were put through a series of ethanol washes with increasing concentrations (50%, 70%, 95%, and 100% ethanol) for one minute each, then two washes with 100% ethanol for two minutes each, and finally, three washes in xylene (Fisher, USA) for two minutes each. Coverslips were mounted with Permount (Fisher, USA) and limb sections were visualized on a Zeiss Axioscop II (Zeiss, Germany).

2.11 – Immunohistochemistry (IHC) on Regenerating Limb Tissue Sections

The following primary antibodies were used for IHC against β -catenin and T β 4 respectively: rabbit monoclonal anti- β -catenin (E247; Abcam, USA) and rabbit polyclonal anti-T β 4 (FL-44; Santa Cruz Biotechnology, USA). The anti- β -catenin antibody was used at 1:350 while the anti-T β 4 antibody was used at 1:200. Supernatant from a mouse hybridoma cell line was used at 1:5 as an anti-MF20 primary antibody to detect myosin heavy chain (MHC, a mature muscle marker). Secondary antibodies were anti-rabbit Cy3/IgG (Jackson ImmunoResearch Laboratories, USA) used at 1:500 and anti-mouse AlexaFluor 488/IgG (Invitrogen, USA) used at 1:400.

To stain the tissue sections, frozen slides were allowed to return to room temperature before being dipped in distilled water and laid flat to allow sections to rehydrate for two minutes. The sections were washed in tris-buffered saline (TBS; see [Appendix III](#)) for three minutes and then blocked with 1% bovine serum albumin (BSA)/5% goat serum (New England Biolabs, USA/Jackson ImmunoResearch, USA) in

TBS for 30 minutes. Primary antibodies (*i.e.*, one of the rabbit antibodies and the mouse-MF20) and 0.3% Triton-X 100 (Sigma, USA) were added to the blocking solution and sections were incubated with this solution overnight at 4°C in a humidified chamber. The following day, sections were washed for two minutes in three changes of TBS and the secondary antibodies (diluted in TBS) were added for one hour at room temperature in a humidified chamber. Sections were again washed for two minutes in three changes of TBS, with DAPI (Invitrogen, USA) being used at 1:1000 in the first wash to counterstain nuclei. Finally, slides were mounted with antifade (see [Appendix III](#)) and sealed with nail polish. Sections were visualized on a Zeiss M2 microscope (Zeiss, Germany).

2.12 – Western Blotting

To verify that the antibodies used for IHC were specifically identifying the corresponding newt proteins, Western blots were performed. Newt limbs were amputated as described above (see [Section 2.2](#)) to collect R0 and R7 tissues. The tissues were digested on ice using RIPA lysis buffer (see [Appendix III](#)) containing protease inhibitors (Roche, Germany). The tissues were homogenized with an Eppendorf pestle (Eppendorf, Germany) followed by passage through a 20 gauge needle. The samples were then sonicated with brief pulses using a Microson Ultrasonic Cell Disrupter XL-2000 (Misonix, USA). Samples were rocked at 4°C for one hour followed by centrifugation for 15 minutes at 4°C and 13000 rpm in a Sorvall Legend Micro 21 R benchtop centrifuge (Thermo Scientific, USA). Protein quantification was performed using the BioRad DC Protein Assay Kit (BioRad, USA). In short, reagents A' and B were added sequentially to the protein samples (including standards). The samples were

incubated at room temperature for 15 minutes and the absorbances were read at 750nm on a Beckman Coulter DU-800 Spectrophotometer (Beckman Coulter, USA).

SDS/PAGE (polyacrylamide gel electrophoresis) was performed by running approximately 30µg of each protein sample (*i.e.*, R0 and R7) through a 4% stacking gel (see [Appendix III](#)) followed by a 10% separating gel (see [Appendix III](#)). Two ladders were run with the samples: 10µl of the BioRad Precision Plus Protein Dual Colour Standard Ladder (BioRad, USA) and 10µl of PageRuler Prestained Protein Ladder (Thermo Scientific, USA). Proteins were transferred overnight onto Immobilon P membranes (Millipore, USA) and membranes were washed with block solution [5% powdered milk (Great Value, USA) in PBS-T (see [Appendix III](#))] for 2.5 hours. The primary antibody [rabbit monoclonal anti-β-catenin (E247; Abcam, USA)] was diluted in block solution (1:2500) and incubated with the blots overnight at 4°C. The following day, blots were washed with PBS-T and incubated with the secondary antibody [anti-rabbit IRDye 800CW (LI-COR, USA)] at 1:10000 for one hour. Following binding of the secondary antibody, the blots were washed again in PBS-T and visualized on an Odyssey CLx Infrared Imaging System (LI-COR, USA).

2.13 – In Situ Hybridization (ISH) on Regenerating Limb Tissue Sections

Limbs were amputated, collected and processed for sectioning as described for above (see [Section 2.9](#)), except that the decalcification process was carried out in RNase-free conditions.

Sense and anti-sense RNA probes were made for *Tβ4* and *Tβ10/15* by conducting PCR on genomic DNA and cloning the amplicon into pGEM-T Easy vector (Promega,

USA) for forward and reverse transcription. In short, genomic DNA was isolated as described above (see [Section 2.7](#)). Primers (see [Appendix II](#)) were supplied by Operon (USA). PCR was carried out using the same protocol as described for colony PCR (see [Appendix I](#) and [Section 2.5](#)). PCR reactions were cleaned as above (see [Section 2.5](#)) using microCLEAN (Gel Company, USA) and amplicons (*i.e.*, probe sequences) were cloned into pGEM-T Easy vector (see [Section 2.5](#)). Plasmids containing the inserts were sequenced (Stem Core Laboratories, Canada) to determine the direction of transcription for sense and anti-sense probes and then sequences were amplified using a combination of M13F/M13R and SP6/T7 primers (see [Appendix II](#)) to make amplicons for run-off transcription.

RNA probes were transcribed and hybridization was performed using a protocol modified from the laboratory of Dr. Valerie Wallace. Briefly, probes were transcribed using 10x Transcription Buffer and RNase OUT from the SuperScript III First-Strand cDNA Synthesis Kit (Invitrogen, USA), 10x DIG-dUTP (Roche, Germany), and either T7 or SP6 RNA polymerase (Roche, Germany) using the run-off amplicons as a template. The mixture was incubated for one hour at 37°C and polymerase was spiked and incubated for an additional hour. RNA probes were ethanol precipitated by adding 0.1 volumes of 3M sodium acetate (pH 5.2; EMD, USA) followed by 2.2 volumes of ice cold 100% ethanol (Commercial Alcohols, Canada). The mixture was placed at -20°C for two hours and then spun for ten minutes at 14000xg in a Sorvall Legend Micro 21 R benchtop centrifuge (Thermo Scientific, USA). The supernatant was removed and the pellet washed with 70% ethanol, followed by another five minute centrifugation. The

supernatant was again removed and the pellet allowed to air dry before resuspending the probe in RNase-free water and storing at -80°C.

Limb sections were post-fixed with 4% PFA (Sigma, USA) for four minutes to better adhere them to the slides. Probes were diluted in hybridization buffer (see [Appendix III](#)) to 1:1000 and denatured at 65°C for ten minutes. Probes were pipetted onto the limb sections and slides were incubated at 65°C overnight in a humidified chamber (humidified with 50% formamide/1X salt; Invitrogen, USA/see [Appendix III](#)). The following day, slides were washed with 65°C wash buffer (see [Appendix III](#)) once for 15 minutes and twice for 30 minutes (at 65°C) followed by two 30 minute washes in 1x MABT (see [Appendix III](#)). Blocking solution (see [Appendix III](#)) was added to sections and incubated at room temperature for one hour in a humidified chamber (humidified with water). Sections were then incubated at 4°C overnight in a humidified chamber with Anti-Digoxigenin-AP Fab Fragments (Roche, Germany) diluted 1:1500 in blocking solution. The next day, slides were washed five times (20 minutes each) with 1x MABT at room temperature. Slides were then equilibrated with staining buffer (without NBT and BCIP; see [Appendix III](#)) for two, ten minute washes at room temperature. Staining buffer with 4.5µl/ml NBT and 3.5µl/ml BCIP (Roche, Germany) was then added to the slides and they were incubated overnight at 37°C in the dark to develop the colour reaction. Staining was stopped by washing the slides several times in PBS (see [Appendix III](#)) and coverslips were mounted with 1:1 glycerol:PBS (Fisher, USA/see [Appendix III](#)) and sealed with nail polish. Limb sections were visualized on a Zeiss M2 microscope (Zeiss, Germany).

2.14 – Culturing and Differentiating A2 and C2C12 Myoblasts

Newt A2 myoblasts were previously isolated in our laboratory from newt limb muscle tissue by Hartej Dhillon. A2s were grown on 10cm dishes, pre-coated with 0.75% gelatin (Anachemia, Canada), in amphibian growth media (AGM, see [Appendix III](#)), with media changes approximately every three to four days. Incubators were maintained at 3% CO₂ and 25°C and myoblasts were passaged after reaching approximately 70-80% confluency to prevent premature differentiation. To induce differentiation, myoblasts were grown to 100% confluency and switched to amphibian differentiation media (ADM, see [Appendix III](#)) for four days, with daily media changes to remove dead cells.

Mouse C2C12 myoblasts were grown on uncoated 10cm dishes in mammalian growth media (GM, see [Appendix III](#)), with media changes approximately every two days. Incubators were maintained at 5% CO₂ and 37°C and myoblasts were passaged similarly to A2s. To induce differentiation, myoblasts were grown to 100% confluency and switched to mammalian differentiation media (DM, see [Appendix III](#)) for four days, with daily media changes to remove dead cells.

For experiments comparing various stages of differentiation, the following criteria were used: subconfluent myoblasts (SCMB) were myoblast cultures which were approximately 50-70% confluent at the time of harvest, confluent myoblasts (CMB) were myoblast cultures which had reached 100% confluency the day before harvesting, and myotubes (MT or 96 hour MT) were cultures which had reached 100% confluency and had been in DM/ADM for four days before harvesting. For some experiments, cells were sampled at various points throughout myotube differentiation (*e.g.*, 24 and 48 hours post

induction of differentiation). For these samples, myoblasts were cultured to 100% confluency in GM and then switched to DM for either 24 hours (24 hour MT) or 48 hours (48 hour MT) before harvesting.

2.15 – Transfection of C2C12 Myoblasts

To determine the functional role of the newt genes during differentiation and dedifferentiation, C2C12 myoblasts were transfected with vectors expressing the *N. viridescens* version of *β-catenin*, *wntless*, *dapper*, and *Tβ4* by Dr. Alan Mears. This work was intended to be completed on A2 myoblasts, which exhibit a more obvious dedifferentiation response. However, due to the difficulty of transfecting these cells, the experiments were done in the faster growing C2C12 myoblasts, which are more amenable to transfection.

The newt versions of *β-catenin*, *wntless*, *dapper*, and *Tβ4* were cloned into the pCI-Neo vector (Promega, USA) at the *EcoRI* site. The plasmids were transfected into C2C12 myoblasts using the Turbofect Transfection Reagent (Fermentas, USA) following the manufacturer's instructions. Transfected cells were selected for neomycin resistance by treating with G418 (Calbiochem, USA). Once selected, cells were maintained under normal culture conditions (see [Section 2.14](#)) with 0.4mg/ml G418.

2.16 – Growth Analysis of Transfected C2C12 Myoblasts (Proliferation Assay)

An equal number of C2C12 myoblasts ($\sim 2 \times 10^4$ cells) overexpressing the newt version of *β-catenin*, *wntless*, *dapper*, or *Tβ4* (or empty pCI-Neo vector as a control) were seeded onto culture plates (35mm or 10cm). For 35mm plates, two plates of cells

per time point (24, 48, and 72 hours) were cultured normally and harvested on the appropriate day. The two plates for each time point were combined and counted, using a Neubauer haemocytometer (Assistent, Germany), to determine if the exogenous genes had any effect on the proliferation rate of transfected C2C12 myoblasts. For 10cm plates, one plate of cells per time point was cultured normally and harvested on the appropriate day. Once again, cells were counted using a Neubauer haemocytometer. For one experiment, an aliquot of the harvested cells was combined with 0.4% Trypan Blue solution (Sigma, USA) and counted with a TC20 Automated Cell Counter (BioRad, USA), to assess the effect of the exogenous genes on proliferation rate and to test for cell death.

2.17 – Differentiation Analysis of Transfected C2C12 Myoblasts (IHC, Fusion Index and Western Blotting)

To assess the effect of overexpression of newt *β-catenin*, *wntless*, *dapper*, and *Tβ4* on the differentiation of C2C12 myoblasts, an IHC for myosin heavy chain (MHC, a mature muscle marker) was performed to compare the formation of myotubes during differentiation among these cell lines. Briefly, myoblasts were grown on 10cm plates and were fixed at the appropriate stage (SCMB, CMB, 24 hour MT, 48 hour MT, or 96 hour MT; see [Section 2.14](#)) with an ethanol/glycine mixture (see [Appendix III](#)) at -20°C for 20 minutes. Immunohistochemistry was performed as above (see [Section 2.11](#)), with the exception that these experiments were performed in 12-well culture plates. For MHC staining, a rabbit polyclonal anti-MYH primary antibody (H-300; Santa Cruz Biotechnology, USA) was used at 1:50 and the secondary antibody was anti-rabbit

Cy3/IgG (Jackson ImmunoResearch Laboratories, USA) used at 1:500. Cells were visualized on a Nikon TE2000 Inverted microscope (Nikon, USA).

To determine the effect of the overexpressed newt genes on C2C12 differentiation, a fusion index was calculated for the 96 hour MT time point for each cell line. The total number of cells was counted per image (via DAPI staining) and the number of nuclei incorporated into MHC positive cell bodies (both multinucleate and mononucleate) was counted. The percentage of total nuclei within multinucleate, MHC positive cell bodies represented the fusion index for a particular cell line.

For the differentiation assay, Western blots were also performed to verify the expression of MHC in the C2C12 samples overexpressing the newt genes. The protocol was similar to that described above (see [Section 2.12](#)) with the following changes: protein was isolated from cells in 10cm plates using CelLytic M Cell Lysis Reagent (Sigma, USA) and protein quantification was read on a Synergy HT multidetection microplate reader (BioTek, USA). Membranes were blocked for one hour in a block solution containing 5% BSA (New England BioLabs, USA) and primary antibodies were a rabbit polyclonal anti-MYH (H-300; Santa Cruz Biotechnology, USA) used at 1:200 and a mouse anti-GAPDH (Abcam, USA) used at 1:15000. An additional anti-mouse IRDye 800CW (LI-COR, USA) secondary antibody was used at 1:10000 to detect the GAPDH antibody.

2.18 – Statistical Analysis

Data were analyzed for statistical significance using either a one-way analysis of variance (ANOVA) with Tukey's post-hoc test or a two-tailed Student's T-Test, assuming unequal variance, as indicated in the figure legends. All statistical analyses

were performed using Graphpad Prism 5.0 software or the Graphpad Software QuickCalcs Online T-Test Calculator (<http://graphpad.com/quickcalcs/ttest1.cfm>). A p-value of <0.05 was considered significant for all statistical analyses.

RESULTS

3.1 – RDA

In order to identify genes involved in early dedifferentiation, an RDA screen was conducted comparing mRNA expression in the intact forelimb (R0) to the three day (R3) regenerate. Genes that were up- and downregulated at three days post-amputation were cloned into pCR4-TOPO or pGEM-T Easy plasmids and plated on LB agar. Approximately 1200 colonies were picked (~600 each from the upregulated and downregulated libraries), colony PCR was performed, and amplicons were sent for sequencing (Operon, USA). The sequences obtained were pared down by first comparing the clones amongst themselves (using BLAST; <http://blast.ncbi.nlm.nih.gov/>) to eliminate multiple copies of the same clone. We then compared our clone database against the Newtomics database (Bruckskotten *et al.*, 2012 and Looso *et al.*, 2013; <http://newtomics.mpi-bn.mpg.de/index.php>), which currently represents genes from intact and regenerating newt heart. Comparing our database to the Newtomics database allowed us to further eliminate multiple copies of our clones and extend sequences whenever possible. Following the elimination of duplicate clones, a list of approximately 410 unique upregulated and downregulated genes (including known *N. viridescens* genes and new potential homologues; [Table 1](#)) were identified. Of the approximately 410 clones screened against the Newtomics database, 209 (~51%) matched or partially matched Newtomics entries, indicating at least half of our genes may have shared roles in limb and heart regeneration. [Note: A recent manuscript (Looso *et al.*, 2013) has examined

Table 1. Approximately 410 unique, differentially regulated genes identified during dedifferentiation in the regenerating newt limb. Genes were identified following RDA comparing R0 to R3 regenerates and subsequent sequencing of clones. The ~1200 clones were reduced to ~410 unique genes through a process of comparing the sequences amongst themselves (using BLAST; <http://blast.ncbi.nlm.nih.gov/>) and against the Newtomics database (<http://newt-omics.mpi-bn.mpg.de/index.php>).

	Known/Potential Homologue	Unknown	Total
Upregulated	167	77	244
Downregulated	103	63	166
Total Number of Genes:			410

gene expression in newt embryonic and larval development, various undamaged tissues, and regenerating limb, tail, and lens. However, the online database has not yet been updated with the genes identified in this study so it is unknown how many of our clones match the most recent data set.]

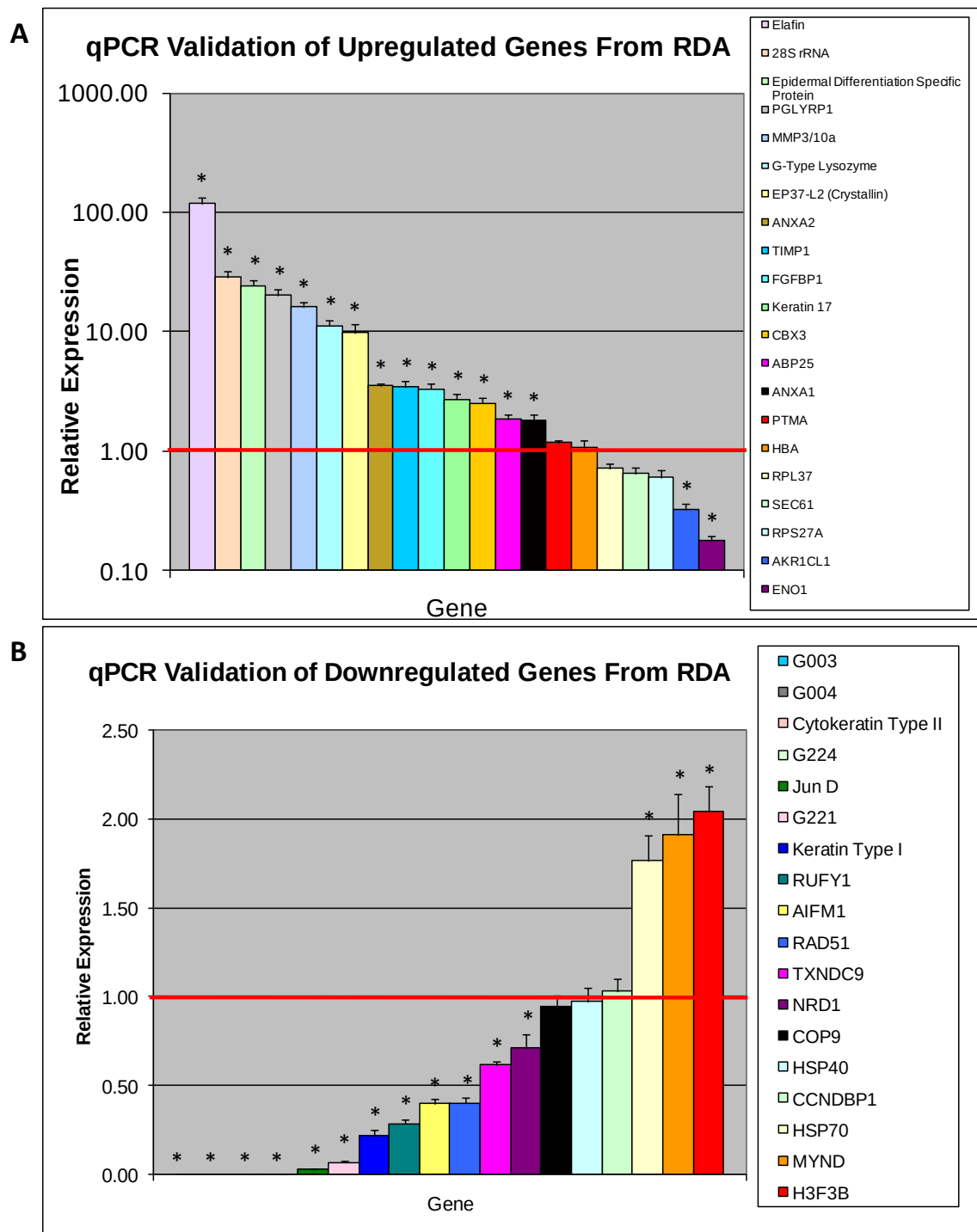
3.2 – Validation of RDA via qPCR

In order to determine if the RDA was successful in identifying differentially regulated genes, a number of genes were chosen for validation and primers (Operon, USA) were designed for qPCR analysis. Of the 39 genes chosen for this analysis, 26 (67%) showed qPCR expression profiles that matched the RDA (*i.e.*, genes which appeared in the upregulated library had upregulated expression following qPCR on R3 cDNA; [Fig. 6](#)). This provided preliminary evidence that the RDA procedure had worked.

3.3 – Validation of RDA and Further Analysis via Microarray

Microarrays were printed in order to perform a larger scale validation of the gene libraries as well as to begin identifying potential candidate genes for further study. Each clone was printed in quadruplicate and microarrays were hybridized with cDNA (transcribed from mRNA) from various stages of limb regeneration (R0, R1, R3, R7, and R21) and tail regeneration (T0, T3, T7, and T15). It is important to note here that the microarray analysis is a much broader and less specific technique than, for example, qPCR. For instance, multiple family members in a cDNA sample, could bind to a single, large clone on the array which would provide a very high signal, but not necessarily specific to the intended family member. Initial hybridization analysis with R0 and R3 cDNA provided high validation rates (~56% and ~65%, respectively) at a very

Figure 6. RDA results validated by qPCR analysis of select genes. (A) Relative expression of select upregulated genes from the RDA following qPCR validation using R3 cDNA template. (B) Relative expression of select downregulated genes from the RDA following qPCR validation using R3 cDNA template. Expression was normalized to *GAPDH* and compared to R0 control (red line). In total, ~67% of the tested genes (26 out of 39) were up- or downregulated as expected from the RDA. cDNA templates were pooled from ≥ 10 newts and qPCR was performed in triplicate (Student's T-Test, $*p < 0.05$ for technical replicates; error bars represent SEM).



stringent p-value of 0.001, further confirming that the RDA procedure was indeed successful at identifying differentially regulated genes ([Fig. 7](#)). Analysis of the remaining time points in both limb and tail provided a picture of the differential expression of the genes printed on the arrays throughout limb/tail regeneration. Clones which did not meet the minimum requirements for signal intensity and p-value were removed from the analysis, providing the final data tables of ~193 known/putative genes ([Table 2](#)) and ~114 unknown genes ([Table 3](#)).

The information from the microarray analysis allows us to identify interesting expression patterns which might indicate a potential gene candidate for follow up. As mentioned above, however, there are limits to the microarray technique: for example, several genes whose expression was validated by qPCR were excluded from the microarray list while some genes from the microarray list were not validated by qPCR. This reiterates that the microarray list is not a complete list of genes, but rather, a tool with which to help identify interesting candidates.

3.4 – RACE on Candidate Genes

Due to the fact that several members of the wnt signalling pathway appeared as potential genes of interest following the RDA (*e.g.*, *wntless*, *β -catenin*, *dapper*) and given that previous work in other regenerating organisms has indicated the importance of this pathway for regeneration (Adell *et al.*, 2009; Kawakami *et al.*, 2006; Lin and Slack, 2008; Yokoyama, 2008; Yokoyama *et al.*, 2007), we decided to test the role of genes of the wnt signalling pathway as regulators of dedifferentiation in the newt.

Figure 7. RDA results validated by microarray analysis. Representative image of microarray analysis (hybridized with R0 cDNA). Printed genes were based on RDA analysis, previous work from our lab, and other known, published newt sequences. The image shows that the majority of genes identified as downregulated by the RDA (*i.e.*, genes identified in the R0 sample which make up the top half of each array) fluoresce red, indicating high expression in the R0 sample. The majority of genes on the bottom half of each array, which were identified as upregulated by the RDA (*i.e.*, identified in the R3 sample), fluoresce green, indicating they are not present in the R0 (downregulated gene) sample. Inset: magnified view of two arrays, indicating each gene is represented by four spots.

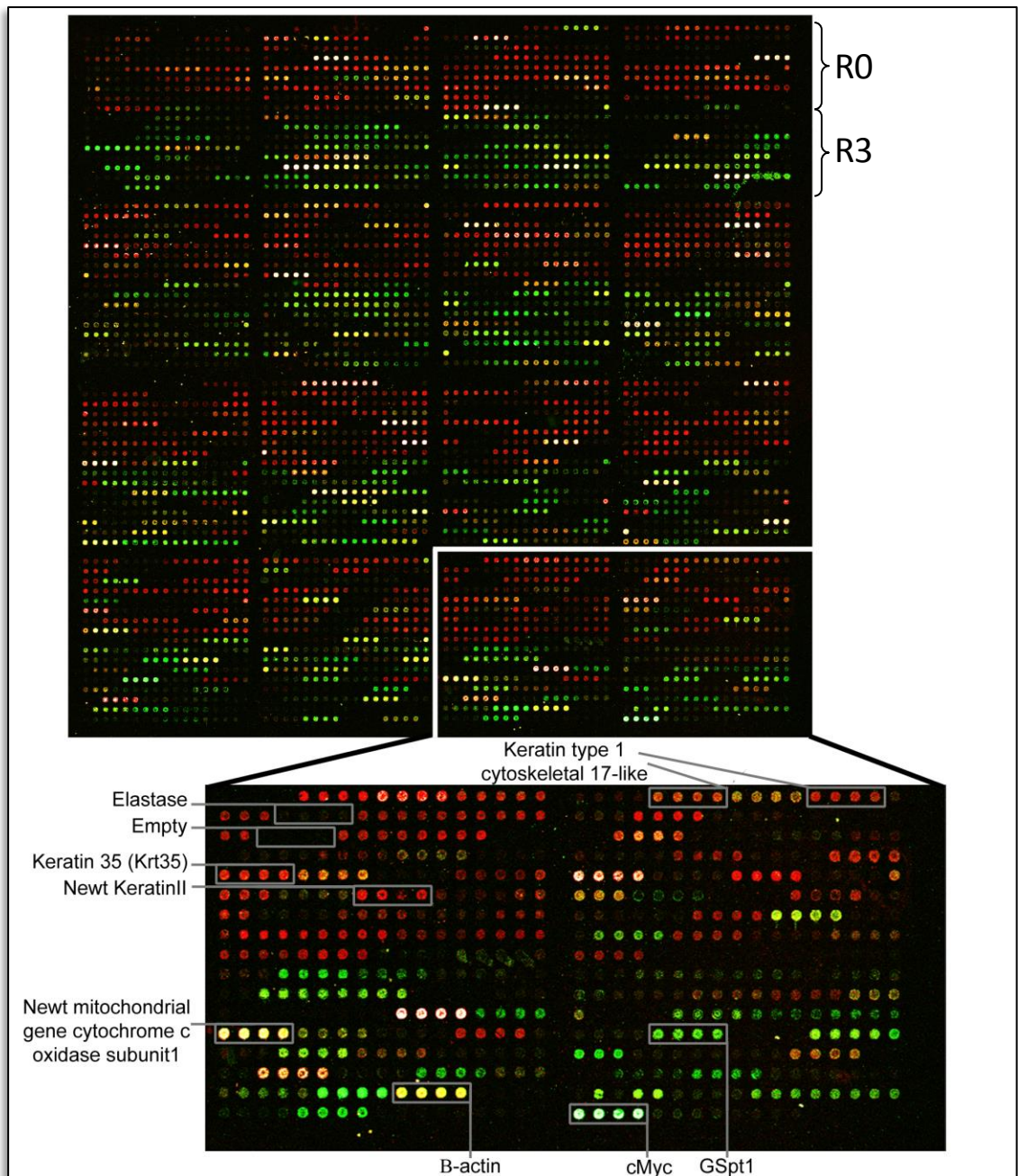


Table 2. Heat map of known/putative genes and their limb/tail expression profiles based on microarray analysis. Fold change (FC) is shown for regeneration in the limb (R) or tail (T) at the indicated day post-amputation (see legend below for heat map colour reference).

up >4-fold
up >2-fold
up <2-fold
no difference
down <2-fold
down >2-fold
down >4-fold

Putative identity	Symbol	Limb Regenerate (FC)				Tail Regenerate (FC)		
		R1	R3	R7	R21	T3	T7	T15
Diamine acetyltransferase 1		-3.5	-5.1	-5.3	-2.9	1.0	1.0	1.0
Staphylococcal nuclease domain containing 1		-2.9	-4.6	-3.5	-4.9	1.0	1.0	1.0
Keratin, type I cytoskeletal 42-like	Krt, I, 42-like	-2.9	-3.2	-3.1	-3.1	1.0	-2.0	1.0
Type II keratin	NvKII	-3.1	-3.6	-3.1	-2.5	1.0	1.0	1.0
Keratin, type I cytoskeletal 47 kDa-like		-2.7	-2.4	-2.9	-3.0	1.0	-1.5	1.0
Cytokeratin type II		-2.4	-3.2	-2.7	-2.0	1.0	1.0	1.0
Keratin 40	Krt40	-1.9	-2.2	-2.8	-2.8	1.0	1.0	1.0
Keratin 5, gene 5	Krt5.5	-2.4	-1.9	-5.8	-2.2	1.0	1.0	1.0
Otokeratin		-2.8	1.0	-3.8	-3.0	1.0	1.0	1.0
Keratin, type I cytoskeletal 14-like		1.0	-2.4	-3.3	-2.1	1.0	1.0	1.0
DnaJ (Hsp40) homolog subfamily B member 9-like		-1.3	-2.4	-2.3	-1.7	1.0	1.0	1.0
S100-A4-like isoform 1		-1.7	-2.2	-3.3	1.0	1.0	1.0	1.0
IMAGE clone 6877235		-1.7	-2.3	-1.9	1.0	1.0	1.0	-1.7
Ribosomal protein S8	Rps8	-1.5	-1.4	-1.7	-1.5	1.0	1.0	-1.7
Ribosomal protein S6	Rps6	-1.4	-1.7	1.0	-1.6	1.0	1.0	-1.6
Keratin 24	Krt24	1.0	-1.7	-3.8	-1.5	1.0	1.0	-1.9
Keratin, type I cytoskeletal 17-like	Krt, I, 17-like	1.0	-1.6	-4.0	1.0	1.0	-7.3	1.0
Lactoperoxidase	Lpo	1.0	1.0	-5.5	1.0	1.0	-3.1	1.0
Cystatin A1		1.0	1.0	-3.9	1.0	1.0	-2.9	-1.9
Keratin 24, variant 2	Krt24	1.0	1.0	-3.4	1.0	1.0	1.0	1.0
Ep37-A2 (crystallin)		2.6	-2.8	-2.8	3.9	-2.5	-3.0	-7.1
Major facilitator family transporter	Tsga	-3.4	1.0	1.0	1.0	1.0	1.0	1.0
Estrogen receptor beta 2	Erb2	1.0	1.0	1.0	-3.0	1.0	1.0	1.0
Ribosomal protein S20	Rps20	1.0	-1.6	-1.6	1.0	1.0	1.0	-1.7
60S ribosomal protein L13a-like		1.0	-1.4	-1.7	1.0	1.0	1.0	-1.5
Ribosomal protein S24	Rps24	1.0	-1.3	-1.8	1.0	1.0	1.0	1.0
Myelocytomatosis oncogene	Myc	1.4	-1.4	-1.7	1.0	1.0	1.0	1.0
Keratin, type I cytoskeletal 12-like		1.0	1.0	-2.3	1.0	1.0	-3.6	1.0
Anaphase-promoting complex subunit 11-like		1.0	1.0	-2.1	1.0	1.0	1.0	1.0
F-box only protein 18-like		1.0	1.0	-1.7	1.0	1.0	1.0	1.0
Actin related protein 3	Actr3	1.0	-1.5	-1.8	1.6	1.0	-2.6	1.0
GLI pathogenesis-related 2	Glpr2	1.0	1.0	-2.0	1.6	1.0	1.0	1.0
Beta-2-microglobulin precursor		1.0	1.0	-1.7	1.7	1.0	1.4	1.0
Glutathione S-transferase 3	Gst3	1.0	1.0	-1.8	2.1	1.0	-1.5	1.0
Actin, alpha, cardiac muscle 1	Actc1	1.0	1.0	-1.4	1.6	1.0	1.0	1.0
T-box 5	Tbx5	2.2	1.0	-1.7	1.0	1.0	1.0	1.0
Wiz protein (widely interspaced zinc finger motifs)	Wiz	1.9	1.0	-1.5	1.0	1.0	1.0	1.0
Keratin 5, gene 2	Krt5	1.5	1.0	-1.8	1.5	1.0	-2.9	1.0
Kruppel-like factor 4	Klf4	1.6	1.0	-1.6	1.4	1.0	1.0	1.0
Ribosomal RNA 12S		1.8	-1.5	-1.5	1.7	1.0	-2.4	1.0
ADP/ATP translocase 2	Adt2	1.0	-1.6	1.0	1.0	1.0	1.0	1.0
Cytochrome c oxidase subunit 3	Cox3	1.0	-1.5	1.0	1.0	1.0	1.0	1.0
Elongation factor 1 delta 2	Eef1d	1.0	-1.6	1.0	1.4	1.0	1.0	1.0
Integrin alpha 2 subunit	Itga2	1.0	1.0	1.0	1.3	1.0	1.0	1.0
Btg2-like	Btg2l	1.0	1.0	1.0	1.5	1.0	-1.8	1.0
Jun D proto-oncogene	Jund	1.0	1.0	1.0	1.6	1.0	1.0	1.0
Epithelial membrane protein 1-like		1.4	1.0	1.0	1.0	1.0	1.0	1.0
Eukaryotic translation initiation factor 3 subunit B-like	Eif3b	1.4	1.0	1.0	1.0	1.0	1.0	1.0
V-ets erythroblastosis virus E26 oncogene homolog 2	Ets2	1.5	1.0	1.0	1.0	1.0	-1.7	1.0
Protein phosphatase 1, catalytic subunit, beta isozyme	Ppp1cb	1.5	1.0	1.0	1.0	1.0	1.0	1.0
Polymerase (RNA) II (DNA directed) polypeptide E, 25kDa	Polr2e	1.5	1.0	1.0	1.0	1.0	1.0	1.0
Galectin family xgalectin-IIa	xgalectin-IIa	1.6	1.0	1.0	1.0	1.0	1.0	1.0
Programmed cell death 8	LOC10022656	1.6	1.0	1.0	1.0	1.0	1.0	1.0
Troponin C, type 2 (fast)	Tnnc2	1.6	1.0	1.0	1.0	1.0	-1.9	1.0
Centaurin, delta 2	Arap1	1.4	1.4	1.0	1.0	1.0	1.0	1.0
Thioredoxin domain-containing protein 9-like		1.5	1.4	1.0	1.0	1.0	1.0	1.0
Matrix metalloproteinase 10b	Mmp10b	1.6	1.4	1.0	1.0	1.0	1.0	1.0
Lipase maturation factor 2-like		2.0	1.0	1.0	1.0	1.0	1.0	1.0
Alpha globin	Hba	2.3	1.0	1.0	1.0	-2.9	-1.9	-10.6
KIAA0652		2.7	1.0	1.0	1.0	1.0	2.0	1.0
Radical fringe	Rfng	1.0	1.4	1.0	1.0	1.0	1.0	1.0
Wntless homolog	Wls	1.0	1.4	1.0	1.0	1.0	1.0	1.0
Interleukin enhancer binding factor 2	Ilf2	1.0	1.5	1.0	1.0	1.0	1.0	1.0
NADH dehydrogenase (ubiquinone) Fe-S protein 3, 30kDa (NADH-coenzyme Q reductase)		1.0	1.5	1.0	1.0	1.0	1.0	1.0
DNA repair protein RAD51 homolog 4-like	Rad51d	1.0	1.5	1.0	1.0	1.0	1.0	1.0
F-box/WD repeat-containing protein 11-like	Fbxw11	1.0	1.8	1.0	1.0	1.0	1.0	1.0
Titin-like		1.0	2.2	1.0	1.0	1.0	1.0	1.0
ADP-ribosylation factor GTPase-activating protein 3-like	LOC10055371	1.0	2.3	1.0	1.0	1.0	1.0	1.0
Aldo-keto reductase family 1, member C-like 1	Akr1c1	1.0	1.4	1.0	1.4	1.0	1.0	1.0
Family with sequence similarity 96, member B	Fam96b	1.0	1.6	1.0	1.4	1.0	1.0	1.0
Fibroblast growth factor binding protein 1	Fgfbp1	1.0	1.5	1.0	1.7	1.0	1.0	1.0
Cystatin A	Csta	1.0	1.8	1.0	1.6	1.0	1.8	1.0
SRY-box containing gene 2	Sox2	1.0	1.6	1.5	1.0	1.0	1.0	1.0

Putative identity	Symbol	Limb Regenerate (FC)				Tail Regenerate (FC)		
		R1	R3	R7	R21	T3	T7	T15
Integrin, beta 1 precursor		1.0	2.0	1.7	1.0	1.0	1.0	1.0
MGC89853 RNA		1.0	-1.9	-1.3	2.0	1.0	-1.5	-2.0
Ubiquitin-40S ribosomal protein S27a-like		1.0	-2.0	1.0	1.9	-2.1	-1.5	-1.8
Keratin 14	Krt14	1.0	1.0	-1.7	3.2	1.0	-2.3	1.0
Ribosomal protein L12	Rpl12	1.0	1.0	1.0	1.8	1.0	1.0	1.0
Ribosomal protein SA	Rpsa	1.0	1.0	1.0	1.9	1.0	-1.4	1.0
Plasminogen activator inhibitor 2-like	LOC10054438	1.0	1.0	1.0	2.0	1.0	1.0	1.0
Ribosomal protein L9	Rpl9	1.0	1.0	1.0	2.1	-1.7	-1.8	1.0
Malate dehydrogenase 1, NAD (soluble)	Mdh1	1.0	1.0	1.0	2.2	1.0	1.0	1.0
Keratin-like	Krt1	1.0	1.0	1.0	2.4	-2.2	-3.0	-3.1
Triose phosphate isomerase	Tpi	1.0	1.0	1.0	2.7	1.0	-2.0	-1.7
Glyceraldehyde-3-phosphate dehydrogenase	Gapd	1.0	1.0	1.0	2.9	1.0	-1.4	-1.7
Chromobox homolog 3	Cbx3-b	1.0	1.5	1.0	2.1	1.0	1.0	1.0
Cold inducible RNA binding protein	Cirbp-b	1.0	1.4	1.0	2.4	1.0	1.0	1.0
Ribosomal protein L37	Rpl37	1.4	-1.6	1.0	2.1	-1.8	1.0	1.0
Cytochrome c oxidase subunit 1	Cox1	1.8	-1.4	1.0	2.0	1.0	1.0	1.0
Ribosomal RNA 18S		1.8	1.0	1.0	1.3	1.0	-3.2	1.0
14-3-3 protein, beta/alpha-like		1.8	1.0	1.0	1.6	1.0	-1.5	1.0
Heterogeneous nuclear ribonucleoprotein K	Hnmpk	1.4	1.0	1.0	1.8	1.0	-1.4	1.0
HLA class II histocompatibility antigen gamma chain-like		1.5	1.0	1.0	1.8	-2.1	-1.4	1.0
Eukaryotic translation initiation factor 3, subunit 6 interacting protein	Eif3s6ip	1.5	1.0	1.0	1.7	1.0	1.0	1.0
Leptin precursor		1.6	1.0	1.0	1.9	1.0	1.0	1.0
Mitochondrial carrier homolog 2	Mtch2	1.4	1.0	1.0	2.4	-2.1	-2.0	1.0
tRNA methyltransferase 112 homolog		1.7	1.0	1.0	2.5	1.0	1.0	1.0
Beta globin	Hbb	1.9	1.0	1.0	2.2	1.0	-1.7	-9.6
Myosin binding protein C, slow type	Mybpc1	1.0	1.0	1.6	1.0	1.0	1.0	1.0
Jumonji, AT rich interactive domain 1B	LOC10022012	1.0	1.0	1.6	1.0	1.0	1.0	1.0
Family with sequence similarity 210, member A	Fam210a	1.0	1.0	1.7	1.0	1.0	1.0	1.0
Elastase 1		1.0	1.0	1.9	1.0	1.0	1.0	1.0
Checkpoint protein Hus1	Hus1	1.0	1.0	1.8	1.3	1.0	1.0	1.0
Sad1 and UNC84 domain containing 1	Sun1	1.0	1.0	2.2	1.0	1.0	1.0	1.0
Non-structural polyprotein 1AB		1.0	1.0	2.6	1.0	1.0	1.0	1.0
Desmoplakin	Dsp	1.0	1.0	2.5	1.5	1.0	1.0	1.0
Catenin alpha-1	Ctnna1	1.5	1.0	1.8	1.0	1.0	1.0	1.0
Aldehyde dehydrogenase 3A2	Aldh3a2	1.9	1.0	1.9	1.0	1.0	1.0	1.0
Glutamine synthetase-like		1.6	1.3	2.0	1.0	1.0	1.0	1.0
Pyruvate kinase, muscle	Pkm2	2.0	1.0	2.7	1.0	1.0	-1.8	1.0
Transcription factor AP-2-epsilon-like		1.9	1.8	1.7	1.0	1.0	1.0	1.0
Collagenase 1		1.7	1.8	2.5	1.0	1.0	1.0	1.0
Endoplasmic-like	LOC10054365	1.5	1.7	1.0	1.6	1.0	1.0	1.0
Elongation factor 2	Eef2	1.9	1.6	1.0	1.8	1.0	-1.7	1.0
GTP-binding protein Rheb precursor	Rheb	1.6	1.3	1.0	1.9	1.0	1.0	1.0
Signal sequence receptor, delta	Ssr4	1.6	1.4	1.0	2.4	1.0	1.0	1.0
Keratin, type I cytoskeletal 13-like		1.5	1.8	1.0	2.2	1.0	-1.5	1.0
Cytochrome c oxidase subunit Vb, gene 1	Cox5b1	1.4	1.5	1.6	2.1	1.0	1.0	1.0
ATP-binding cassette sub-family F member 2	Abcf2	1.5	1.5	1.5	2.3	1.0	1.0	1.0
Beta actin	Actb	1.8	1.6	1.5	2.2	1.0	1.0	1.0
Microtubule-associated protein, RP/EB family, member 1	Mapre1	1.5	2.1	1.6	2.0	1.0	1.0	1.0
Influenza virus NS1A-binding protein homolog		2.2	2.2	1.0	1.5	1.8	1.0	1.0
Annexin A1-like	Anxa1l	2.0	2.1	1.0	2.2	1.0	1.0	1.0
S100-A10-like	LOC10001625	1.7	2.5	1.0	2.9	1.0	1.6	1.0
ES1 protein homolog, mitochondrial	LOC422305	2.6	2.2	1.0	2.8	1.0	1.0	1.0
Integrin, beta 1	LOC10023094	2.4	2.0	1.4	2.5	1.0	1.0	1.0
Proteasome subunit alpha type-3	Psa3	1.0	2.0	1.4	1.6	1.0	1.6	1.0
Hypothetical protein LOC100490020		1.0	2.2	1.6	1.6	1.0	1.0	1.0
Chaperonin containing TCP1, subunit 5 (epsilon)	Cct5	1.0	1.7	1.9	1.7	1.0	1.0	1.0
Alpha enolase		1.0	1.8	2.2	1.9	1.0	1.0	1.0
Riddle 4	Ridd4	1.0	2.3	2.0	1.6	1.0	2.3	3.3
Leukocyte cysteine proteinase inhibitor 1		1.0	2.0	1.7	2.3	1.9	1.4	1.0
Costars family protein C6orf115 homolog		1.0	1.9	1.9	2.4	1.0	1.0	1.0
Dihydroliipoamide S-succinyltransferase (E2 component of 2-oxo-g Dlst		1.4	2.6	2.2	1.5	1.0	1.0	1.0
Annexin A2	Anxa2	1.8	2.5	1.9	1.7	1.0	1.0	1.0
Mitogen-activated protein kinase 13	Mapk13	1.7	1.8	2.3	2.0	1.0	1.0	1.0
Lactate dehydrogenase A	Ldha	1.9	2.4	2.3	2.3	1.0	1.0	1.0
SH3 domain protein D19	Sh3d19	1.0	1.8	2.4	1.0	1.0	1.0	1.0
Putative serine proteinase inhibitor		1.0	2.7	2.8	1.0	1.9	1.9	1.0
Collagenase	Col	1.0	2.4	3.0	1.4	1.0	1.0	1.0
Receptor accessory protein 5	Reep5	1.0	2.9	3.0	1.7	1.0	1.0	1.0
Proximal identity 1	Prod1	1.8	2.5	3.4	1.0	1.0	1.0	1.0
Sec61 beta subunit	Sec61b	1.0	1.0	1.5	1.8	1.7	1.0	1.0
Eukaryotic translation initiation factor 3, subunit E	LOC10058295	1.0	1.0	1.7	1.9	1.0	1.0	-1.7
ATP synthase, H+ transporting, mitochondrial F1 complex, epsilon Atp5e		1.0	1.0	1.5	2.8	1.0	-1.5	1.0
Creatine kinase B		1.0	1.0	1.8	2.6	1.0	1.0	1.0
Calmodulin-like		1.4	1.0	1.9	3.0	1.0	-1.4	1.0
High-mobility group nucleosome binding domain 1	Hmgn1	1.0	1.0	1.6	3.8	1.0	1.0	1.0

Table 3. Heat map of unknown genes and their limb/tail expression profiles based on microarray analysis. Fold change (FC) is shown for regeneration in the limb (R) or tail (T) at the indicated day post-amputation (see legend below for heat map colour reference).

up >4-fold
up >2-fold
up <2-fold
no difference
down <2-fold
down >2-fold
down >4-fold

Unique Gene Identity	Limb Regenerate (FC)				Tail Regenerate (FC)		
	R1	R3	R7	R21	T3	T7	T15
G020	-6.2	-33.1	-13.7	-5.6	1.0	1.0	1.0
G157	-12.9	-26.3	-8.7	-5.1	1.0	1.4	3.4
G003	-9.0	-16.0	-11.8	-6.3	1.0	1.0	1.0
G117	-3.6	-18.6	-16.4	-7.7	1.0	1.0	1.0
G037	-5.8	-4.0	-10.4	-4.4	1.0	1.0	1.0
G001	-3.3	-6.8	-7.2	-4.0	1.0	1.0	1.0
G004	-2.7	-6.2	-8.5	-4.1	1.0	1.0	1.0
G230	-2.4	-7.9	-7.1	-4.1	1.0	1.0	1.0
G134	-4.1	-2.8	-4.6	-4.4	1.0	1.0	1.0
G040	-2.7	-3.0	-4.4	-2.9	1.0	1.0	1.0
G008	-2.7	-2.8	-3.2	-3.6	1.0	1.0	1.0
G046	-2.8	-3.2	-1.8	-3.1	-2.4	-2.0	1.0
G016	1.0	-8.2	-20.5	-1.4	-2.3	-13.8	-2.3
G224	-2.6	-3.2	-7.3	1.0	-2.9	-4.2	-4.1
G012	1.0	-6.1	-6.4	1.7	1.0	-3.9	-1.7
G030	-1.7	1.0	-3.7	-2.2	1.0	1.0	1.0
G216	1.0	1.0	-5.4	1.0	1.0	1.0	1.0
G325	-2.0	-1.8	-2.5	1.0	1.0	-1.9	1.0
G221	-1.5	-2.2	-4.5	1.0	1.0	-1.9	-2.0
G199	1.0	-3.6	-3.5	1.0	-2.7	-4.0	-2.1
G220	1.0	-3.9	-2.6	1.0	1.0	-2.5	-1.6
G013	1.0	-2.5	-3.7	1.0	1.0	1.0	1.0
G015	1.0	-2.4	-2.9	1.0	-2.7	1.0	-1.6
G166	1.0	-2.6	-2.4	1.0	1.0	-2.0	1.0
G010	1.0	-1.4	-3.3	1.0	1.0	1.0	-2.0
G158	1.0	-1.6	-2.4	1.0	1.0	1.5	1.7
G011	1.0	-1.6	-1.9	1.0	1.0	1.0	1.0
G186	1.0	-2.9	1.0	1.3	1.0	-1.4	1.0
G071	1.0	-2.2	1.0	1.0	1.0	-1.8	1.0
G081	1.0	-1.6	1.0	1.6	1.0	1.0	1.0
G289	1.0	-2.0	1.0	2.3	1.0	-2.2	1.0
G005	1.0	-2.2	-2.1	3.3	1.0	-3.1	1.0
G140	1.0	-1.4	-1.7	2.2	1.0	-1.8	1.0
G032	1.9	-1.6	-1.6	1.8	1.0	-2.1	-1.7
G131	1.0	1.0	-2.0	1.0	1.0	1.0	1.0
G021	1.0	1.0	-2.0	1.0	1.0	1.0	1.0
G006	1.0	1.0	-1.8	1.0	1.0	1.0	1.0
G028	1.0	1.0	-1.5	1.0	1.0	1.0	1.0
G173	1.0	1.0	-1.4	1.0	1.0	1.0	1.0
G017	1.0	-1.6	1.0	1.0	1.0	1.0	1.0
G031	1.0	-1.4	1.0	1.0	1.0	1.0	1.0
G019	1.0	1.0	1.0	-1.2	1.0	1.0	1.0
G300	1.0	1.5	1.0	1.0	1.0	1.0	1.0
G100	1.0	1.5	1.0	1.0	1.0	1.0	1.0
G109	1.0	1.5	1.0	1.0	1.0	1.0	1.0
G080	1.0	1.7	1.0	1.0	1.0	1.0	1.0
G002	1.0	1.7	1.0	1.0	1.0	1.0	1.0

Unique Gene Identity	Limb Regenerate (FC)				Tail Regenerate (FC)		
	R1	R3	R7	R21	T3	T7	T15
G099	1.0	1.8	1.0	1.0	1.0	1.0	1.0
G023	1.0	1.9	1.0	1.0	1.0	1.7	1.0
G277	1.0	2.0	1.0	1.0	1.0	1.0	1.0
G073	1.0	2.0	1.0	1.0	1.0	1.0	1.0
G101	1.0	2.0	1.0	1.0	1.0	1.0	1.0
G329	1.0	1.9	1.3	1.0	1.0	1.0	1.0
G112	1.0	2.7	1.0	1.0	1.0	1.0	1.0
G185	1.5	1.0	1.0	1.0	1.0	1.0	1.0
G093	1.7	1.0	1.0	1.0	1.0	1.0	1.0
G337	1.7	1.0	1.0	1.0	1.0	1.0	1.0
G162	1.7	1.0	1.0	1.0	1.0	1.0	1.0
G113	1.8	1.0	1.0	1.0	1.0	1.0	1.0
G009	1.7	1.4	1.0	1.0	1.0	1.4	1.0
G323	1.5	1.7	1.0	1.0	1.0	1.0	1.0
G056	1.4	1.0	1.5	1.0	1.0	1.0	1.0
G333	2.0	1.0	1.7	1.0	1.0	1.0	1.0
G174	1.7	1.0	1.5	1.4	1.0	1.0	1.0
G108	1.6	1.0	1.6	1.7	1.0	1.0	1.0
G045	1.0	1.0	1.0	1.6	1.0	-1.6	1.0
G326	1.0	1.0	1.0	1.7	1.0	1.6	1.0
G342	1.0	1.0	1.0	1.9	1.0	1.0	1.0
G307	1.0	1.0	1.0	2.1	1.0	1.0	1.0
G127	1.4	1.0	1.0	1.6	1.0	1.0	1.0
G364	1.0	1.0	1.0	3.4	1.0	1.0	1.0
G027	1.6	1.3	1.0	2.1	1.0	1.0	1.0
G291	1.4	1.2	1.0	3.0	1.0	1.0	1.0
G039	1.0	1.0	1.5	1.0	1.0	-2.0	1.0
G067	1.0	1.0	1.7	1.0	1.0	1.0	1.0
G202	1.0	1.0	1.9	1.0	1.0	1.0	1.0
G070	1.0	1.0	2.0	1.0	1.0	1.0	1.0
G090	1.0	1.0	2.0	1.0	1.0	-1.5	1.0
G060	1.0	1.0	1.9	1.3	1.0	1.0	1.0
G222	1.0	1.8	1.9	1.0	1.0	1.0	1.0
G340	1.0	1.8	2.1	1.0	1.0	1.0	1.0
G104	1.0	2.3	1.9	1.0	1.0	1.0	1.0
G061	1.0	2.0	2.7	1.0	1.0	1.0	1.0
G107	1.4	1.6	2.0	1.0	1.0	1.0	1.0
G042	1.0	1.5	1.6	1.5	1.0	1.0	1.0
G084	1.0	1.6	1.7	1.7	1.0	-2.0	1.0
G106	1.0	1.7	1.9	1.3	1.0	1.0	1.0
G105	1.0	1.9	1.8	1.4	1.0	1.0	1.0
G091	1.0	2.0	2.1	1.3	1.0	1.0	1.0
G053	1.0	2.2	3.1	1.5	1.0	2.1	1.0
G312	1.7	1.5	2.2	1.6	1.0	1.0	1.0
G370	1.4	1.9	2.0	1.9	1.0	1.0	1.0
G322	1.7	1.5	3.2	1.0	1.0	1.0	1.0
G098	1.0	-1.8	2.0	2.1	1.0	-1.8	1.0
G293	1.0	1.0	2.3	2.5	1.0	2.5	1.0

Unique Gene Identity	Limb Regenerate (FC)				Tail Regenerate (FC)		
	R1	R3	R7	R21	T3	T7	T15
G290	1.6	1.6	1.0	3.9	1.0	1.6	1.5
G302	2.5	1.9	1.0	4.2	1.0	1.5	2.9
G120	2.1	2.2	1.8	3.2	1.0	1.0	1.0
G072	1.9	2.8	1.9	1.0	1.0	1.0	1.0
G052	1.5	2.9	1.8	1.6	1.0	1.0	1.0
G082	2.2	2.4	2.1	1.7	1.0	1.0	1.0
G077	2.1	2.9	2.9	1.7	1.8	1.6	1.0
G241	1.4	3.6	2.8	2.9	2.0	1.0	1.7
G254	1.5	2.7	3.8	3.1	1.0	2.0	1.0
G110	1.0	3.7	5.3	1.0	1.0	-1.7	1.0
G286	1.0	2.2	5.2	2.3	1.0	1.0	1.0
G057	1.0	3.1	7.7	1.8	-2.2	-1.4	1.0
G058	2.3	2.4	10.5	2.2	1.0	1.0	1.0
G235	2.1	2.2	10.5	2.8	2.4	2.9	1.7
G086	2.4	5.4	5.3	2.7	1.0	1.0	1.0
G051	3.1	5.4	4.2	3.1	2.1	2.9	1.0
G043	3.6	4.3	7.7	4.6	1.0	4.5	2.4
G055	4.3	12.1	14.6	2.7	1.0	2.2	2.1
G263	4.3	25.0	19.5	1.8	4.0	5.6	6.3

The first step in the process was to clone the full length cDNAs for these genes. The full length coding sequence (CDS) of newt *β-catenin*, *wntless*, and *dapper* were identified using 5'- and 3'-RACE. The original clone for *β-catenin* was 342bp and it was expanded to 3038bp; the original 469bp *wntless* clone was expanded to 1737bp; and the original 556bp *dapper* clone was expanded to 2498bp. [Figures 8, 9, and 10](#) show multiple sequence alignments for *β-catenin*, *wntless*, and *dapper*, respectively, comparing each newt protein to human, mouse, chicken, frog, and fish sequences. Newt *β-catenin* is highly conserved (~98% at the protein level) among vertebrates throughout the coding region ([Fig. 8](#)). Newt *wntless* is also well conserved among vertebrates throughout the coding region (~75% at the protein level; [Fig. 9](#)). Newt *dapper* is less well conserved among vertebrates (~60% at the protein level), becoming particularly divergent towards the N-terminus in *N. viridescens* ([Fig. 10](#)).

Another interesting candidate gene family from the RDA was *thymosin-β* (*Tβ*). Genes from this family (*Tβ4*, in particular) have been shown to be involved in mammalian tissue repair and regeneration and may even be novel regulators of wnt signalling (Goldstein *et al.*, 2012; Ryu *et al.*, 2012). For these reasons, we also decided to test *Tβ4* as a regulator of dedifferentiation in the newt. Dr. Alan Mears originally identified members of this family of genes based on their high expression in the microarray analysis and in the RDA. The original 227bp clone was expanded by RACE (to 605bp) and a BLASTP analysis showed high homology to both human thymosin-β 15 and thymosin-β 10. Since we cannot be sure which of the two genes the newt cDNA represents, we called it *Tβ10/15*. During RACE expansion of this clone, a related family member, *Tβ4*, was also identified (670bp).

Figure 8. Multiple sequence alignment of *N. viridescens* β -catenin protein sequence with that of chicken, mouse, human, frog, and fish. Newt β -catenin (highlighted in yellow) is highly conserved among vertebrates (~98% in the coding region). Alignment performed with ClustalW2 multiple sequence alignment tool (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). [Legend: “*” = all residues in column are identical; “:” = conserved substitutions are observed; “.” = semi-conserved substitutions are observed; **red** = small + hydrophobic (including aromatic –Y); **blue** = acidic; **magenta** = basic – H; **green** = hydroxyl + sulfhydryl + amine + G; **grey** = other]

Chicken	VRAHQDTQRRTS	SMGGTQQQFVEGVRMEEIVEGCTGALHILARDVHNRIVIRGLNTIPLFV	600										
Mouse	VRAHQDTQRRTS	SMGGTQQQFVEGVRMEEIVEGCTGALHILARDVHNRIVIRGLNTIPLFV	600										
Human	VRAHQDTQRRTS	SMGGTQQQFVEGVRMEEIVEGCTGALHILARDVHNRIVIRGLNTIPLFV	600										
Newt	VRAHQDTQRRTS	SMGGTQQQFVEGVRMEEIVEGCTGALHILARDVHNRIVIRGLNTIPLFV	600										
Xenopus	VRAHQDTQRRTS	SMGGTQQQFVEGVRMEEIVEGCTGALHILARDIHNIRIVIRGLNTIPLFV	600										
Zebrafish	VRAHQDTQRRTS	SMGGTQQQFVEGVRMEEIVEGCTGALHILARDIHNIRIVIRGLNTIPLFV	599										
*****.*****													
Chicken	QLLYSPIENIQ	RVAAGVLC	LAQDK	EAAE	IAEAG	TAPL	TELLHS	RNEG	VATY	AAAVLF	660		
Mouse	QLLYSPIENIQ	RVAAGVLC	LAQDK	EAAE	IAEAG	TAPL	TELLHS	RNEG	VATY	AAAVLF	660		
Human	QLLYSPIENIQ	RVAAGVLC	LAQDK	EAAE	IAEAG	TAPL	TELLHS	RNEG	VATY	AAAVLF	660		
Newt	QLLYSPIENIQ	RVAAGVLC	LAQDK	EAAE	IAEAG	TAPL	TELLHS	RNEG	VATY	AAAVLF	660		
Xenopus	QLLYSPIENIQ	RVAAGVLC	LAQDK	EAAE	IAEAG	TAPL	TELLHS	RNEG	VATY	AAAVLF	660		
Zebrafish	QLLYSPIENIQ	RVAAGVLC	LAQDK	EAAE	IAEAG	TAPL	TELLHS	RNEG	VATY	AAAVLF	659		
*****.*****													
Chicken	RMSEDKPQDY	KKRLSV	ELTSSL	FRTE	PMAWN	ETADL	GLDIG	AQGE	PLGY	RQDD	PSYRSFH	720	
Mouse	RMSEDKPQDY	KKRLSV	ELTSSL	FRTE	PMAWN	ETADL	GLDIG	AQGE	ALGY	RQDD	PSYRSFH	720	
Human	RMSEDKPQDY	KKRLSV	ELTSSL	FRTE	PMAWN	ETADL	GLDIG	AQGE	PLGY	RQDD	PSYRSFH	720	
Newt	RMSEDKPQDY	KKRLSV	ELTSSL	FRTE	PMTWN	ETGDL	GLDIG	AQGE	TLGY	RQDD	PSYRSFH	720	
Xenopus	RMSEDKPQDY	KKRLSV	ELTSSL	FRTE	PMPWN	EADL	GLDIG	AQGE	PLGY	RQDD	SSYRSFH	720	
Zebrafish	RMSEDKPQDY	KKRLSV	ELTSSL	FRTE	PMTWN	ETGDL	GLDIG	AQGE	PLGY	RQDD	PSYRSFH	719	
*****.***.*****.*****.*****													
Chicken	SGGYGQDAL	GMDPMME	HEMGGH	HPGADY	PVDGL	PD	LGH	AQDL	MDGL	PPGDS	NQLAWF	DTDL	781
Mouse	SGGYGQDAL	GMDPMME	HEMGGH	HPGADY	PVDGL	PD	LGH	AQDL	MDGL	PPGDS	NQLAWF	DTDL	781
Human	SGGYGQDAL	GMDPMME	HEMGGH	HPGADY	PVDGL	PD	LGH	AQDL	MDGL	PPGDS	NQLAWF	DTDL	781
Newt	SGGYGQDAL	GMDPMME	HEMGGH	PGSDY	PVDGL	PD	LGH	AQDL	MDGL	PPGDS	NQLAWF	DTDL	781
Xenopus	AAGYGQDAL	GMDSMMD	HDMGGH	HPGADY	PVDGL	PD	LGH	AQDL	MDGL	PPGDS	NQLAWF	DTDL	781
Zebrafish	SGGYGQDAL	GMDPMME	HEMAGH	HPGDPY	PVDGL	PD	LGH	TQDL	IDGL	PPGDS	NQLAWF	DTDL	780
:.*****:*.*:*.*****.*****.*****.*****.*****.*****.*****													

Figure 9. Multiple sequence alignment of *N. viridescens* wntless protein sequence with that of mouse, human, chicken, fish, and frog. Newt wntless (highlighted in yellow) is well conserved among vertebrates (~75% in the coding region). Alignment performed with ClustalW2 multiple sequence alignment tool (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). [Legend: “*” = all residues in column are identical; “:” = conserved substitutions are observed; “.” = semi-conserved substitutions are observed; red = small + hydrophobic (including aromatic –Y); blue = acidic; magenta = basic – H; green = hydroxyl + sulfhydryl + amine + G; grey = other]

Figure 10. Multiple sequence alignment of *N. viridescens* dapper protein sequence with that of mouse, human, chicken, fish, and frog. Newt dapper (highlighted in yellow) is somewhat conserved among vertebrates (~60% in the coding region) and is particularly divergent at the N-terminus in the newt. Alignment performed with ClustalW2 multiple sequence alignment tool (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). [Legend: “*” = all residues in column are identical; “:” = conserved substitutions are observed; “.” = semi-conserved substitutions are observed; red = small + hydrophobic (including aromatic –Y); blue = acidic; magenta = basic – H; green = hydroxyl + sulfhydryl + amine + G; grey = other]

Human --MKPSPAGTAKELEPPAPARGEQRTAEPEGRWREKGEADTERQRTREERQEATLAGLAE 58
 Mouse --MKP---DAAREPEPLSPGRG----AAEGRWRERGEADTERQRTREERQEATLAGLAE 51
 Chicken --MKP---SPAAGAAAAGPGLG-QAAGGPDARWREKGEAEERQRTREERLEATLAGLGEL 54
 Xenopus --MKPIPATPDHLGQH-----ESPRRKDKGEAESEFQRTREERLEATLAGLAE 47
 Newt MLKTAIPVALERLQPPESQGMMLKPGMMES*GWREKGEAESEFQRTREERLEATLAGLGEL 60
 Zebrafish --MNEWSEMLVCRDYCALMMRD-----RKECDARCCRDGEGEVRARERLDATVAGLTLE 53
 . . : :. * * : * * : * * : * *

Human EYLRQEQELLVRGALRGAGGAGAAAPRAGELLGAAQSRLEEKFLEENILLRRQLNCL 118
 Mouse GYLRQEQELLVRGALRCSGTVGTVPAPRSGELRGDAAQSRLEEKFLEENILLRRQLNCL 111
 Chicken EYLRQEQELLVKSLLRR-APGAQGG-GEQQEGPPPSLEEKFLEENILLRRQLNCL 112
 Xenopus GYLRHRQELLVKALTPG-----APTHGDTATRAGDTPRSLEEKFLEDNILLKKQLNCL 102
 Newt EYLRHRQEGLVKAALSPG-----VR-----SMARPEPRSEENILEENILLRRQLNCL 109
 Zebrafish EYLRQEQELLVRSVLSCLP-----EAAGQDKPCLTVDDSYLNTTEKLEENILLRRQLNCL 109
 * * : * * : * * : * * : * * : * * : * *

Human RRRDAGLLNQLQELDKQISDLRLDVEKTSEEHLETDSRPSSGFYELSDGASGSLNSSNS 178
 Mouse RRRDAGLLNQLQELDKQISDLRLDVEKTSEEHLETDSRPSSGFYELSDGASGSLNSSNS 171
 Chicken RRRDAGLLNQLQELDKQISDLRLDVEKTTEEHELETDSRPSSGFYELSDGASGSLNSSNS 172
 Xenopus RKRDAAGLLSQLHELDKQINDLKIDVEKT-EEHELETDSRPSSGFYELSDGTSGSLNSSNS 161
 Newt RRRDAGLLTQLHELDKQISDLRLDVEKT-TPNEQNETDSRPSSGFYELSDGTSGSLNSSNS 169
 Zebrafish RRRDAGLLNQLQELDRQISDLRLDTETS-HEHVETDSRPSSGFYELSDGASGSLNSSNS 168
 * : * * * * : * : * * * * : * : * : * : * : * : * : * : * : * : * : * : *

Human VFSECLSSCHSSTCFCSPLEATLSLDGCPKSAD-LIGLLEY-KEGHCEDQASGAVCRSL 236
 Mouse VFSECLSSCHSSTCFCSPLEAALTISDGCPSAD-LIGWLEC-KGGPCEDQASGTVCSSP 229
 Chicken VFSECLSSCHSSTCFCSPLEATLNISDGRPKSAD-LIGWMDYNKESQREDQTAGSVCRSL 231
 Xenopus VFSECLSSCHSSTCFCNPLETSLNLDGQAKSADDFLEWLDY-RDSQ---HEGTGTVRRSF 217
 Newt VFSECLSSCHSSTCFGSPLEASLTIEGPRKSTDDLLDWIDH-KEGQNDQASGVVRRSL 228
 Zebrafish VFSECLSSCRSTTCLCTPLDTSLCASEGRLKPADDPGSCAEC--DCHCEDSSSGTVRRSL 226
 * * * * * : * : * : * : * : * : * : * : * : * : * : * : * : *

Human STPQFNSLDVIADVNPKYQCDLVSKNGNDVYRYPSPHAAVAVQSPMFLCLTGNPLRE-- 294
 Mouse STPQFNSLDVIADVNPKYQCDLVSKNGNDVYRYPSPHAAVAVQSPMFLCLTGNPLRE-- 287
 Chicken STPHSNSLDVADVHPKYQCDLVSKNGSDVYRYPSPHAAVAVQSPMFLLPVTENPQQE-- 289
 Xenopus SAPHNSNSVDVAADVHPKYQCDLVSKNGNDIYRYPSPHAAVAVQSPMFLSVMGNIKAEEP 277
 Newt SAPNSSSSDVADVHPKYQCDLVSKNGGDVYRYPSPHAAVAVQSPMFLSVMGSLK---- 284
 Zebrafish SASYSPSPDSSCDGISKFHCDLIAKNGNDVYRYPSPHAAVAVQSPVFIQSMSTSHLKDD-- 284
 * : . * * * . * : : * * : * * : * * : * * : * * : * * : * * : *

Human EDRLGNHASDICGGSELDVAKTDSSLPSPSSLWSASHPSSSKMDGYILSLVQKTHPVR 354
 Mouse EELGSHASDICIGSELNATKTDNSLPSPSSLWSASHPASSKKMDGYILSLVQKTHPVR 347
 Chicken EERLGCDITDVCATSETDSGKTAFLPPGS-WPASCPSASKRIDGYILSLVQRKTHSVR 348
 Xenopus EEEIGPNDNDDCIPELDHLKDEDSFLHQSSLCSLPLSSG-KKMDGYILSIIQKKAHPVR 336
 Newt EEKLCRTSDACTEPETNTVLESS--SQNNVCNVPCSS--KKLDSYILGVIQKVVHVSF 340
 Zebrafish -----CNFSKPG-EALNDEQKPEQVVGSGTSSWHASQMLPNKKLDSYIFGLLQRRAPLR 338
 . . : * . . . * : * : * : * : * : * : *

Human TNKPRTSVNADPTKGLLRNGSVCVRAPGG-VSQGNSVNLKNSKQACLPSSGGIPSLNNGTF 413
 Mouse TNKPRTSVNADPTKGLLRNGSVCVRAPSG-VPPGSSVNFKNTKQMCLPAGGITSLENGPF 406
 Chicken TNKPRTSVNADPTKGLRHSIMCVQRTAGVSSHSSVNLKSAKQVSLPSSGATASDNTAP 408
 Xenopus TNKPRTSVNADPGKILRHGSIQVKTGG-VSQSSTVNLKNSKQTCCLHSTGMIQVDNSTY 395
 Newt TNKPRTSVNADPSKILRHGSIQVKTSG-VSQSNSANMKNAFTCLHSGGTNALDVSSS 399
 Zebrafish TNKPRTSINTDPSKILRQASLCRAPASVQAQWTSDLKPNWQTCQLQDASATSTEPSTA 398
 * * * * * : * * : * * : * : * : * : * : * : * : *

Human SPPKQWSKESKAEQAESKRVPLEPGCPGSAASDLQSKHLPK-TAKPASQEHARCSAIGTG 472
 Mouse SPPKQSKDSKTDQLESKRLLALPESCSAGAAMEPQSKHVPK-AAKAASQELTRCQAG-LG 464
 Chicken SPLKQRPREAGGEQVESRKVLLPAAFPF---SELQSKHQPR-GAKAVPPELSRHVAATG 464
 Xenopus PSLKQCSKESLSEQLSKRMPISITYPSCNVNELQSQNNRNNTVKSVCQGLARSGSVMTS 455
 Newt -SPKQWSKEQLSEQLSKRMPVSNVYPPSG-NEVPSTNLIRNNVKTLSPLVSS-SPAVTA 457
 Zebrafish SPQRQWSAESKGGTPQN-GAYLSSSQPQNSYSTNDNTNCLLKKKVSQTSKQGLVSATPK 457
 . : * . : : . . . *

Human ESPKE-----SAQLSGASPKEPSRGPAPPQENKVVQPLKKMSQKNSLQGV 519
 Mouse ESMKE-----SNQASAVSPKTSRGPVAPAESKALQLPKKMSQKNSLQAVP 511
 Chicken DVPKE-----GSQHFAASPKEPSRGPVVLQPETRVSQPPKKVLLKGSSQAAH 511
 Xenopus NVQKENVTPNAPTNLNASSSACNGSPRESTQMSALLPQEIKVVPVVKKISPKNTLLS-Y 514
 Newt DVEKTP--TP-----SSVSTAPVKESPCHRYPTSHQENKVIHPPAKIAPNNCRSPY 505
 Zebrafish DCDPLG-----SPKAVSSPKLN-KHCFYNVEDNKTGQAMKASPLKRSPTQT 503
 : . . : : . : : *

Human PATPPLLSTAFPVVEERPALDFKSEGS--QSLEEA-HLVKAQFIPGQQPSVRLHRG-HRNM 576
 Mouse -----ALDRPALDFKSEGS--QSLEEG-HLVKAQFIPGQQAARPHRA-HRNP 556
 Chicken SSSPP-----VEERPALDFKSEGSQSDDG-LLVNAQYIPAQQQSVKLHKG-TRNV 562
 Xenopus HASSS-----FDERPPLDFKSEGSQSDEG-LLVNAHYIPAQQQGVKLHKN-IKNV 565
 Newt QVAPS-----LVERPQLDFKSEGSQSDEG-LLVKGQYIPAQDQGVKLHKG-TKNL 556
 Zebrafish SLSCG-----KDSEQLAQELVSLGSSQSDEGGPLVSAQYIPAQKQNVNQLQGGTKNI 557
 :: :: * ** * :. *. :. :. : *

Human GVVKNSSLKHRGPALQGLENGLPTVREKTRAGSKKCRFPDDL-TNKKLKKASSKGRKSG 635
 Mouse GVARSATLKARG--QAAMEHGLPTVREKPRAAGKKCRFPDDSD-TNKKFRKTSAKGRSG 613
 Chicken KILKSSALKHRPHLANGLENGAALREKGPISKKCRFPDELD-TNKKLKKPSSRGKRGG 621
 Xenopus KIVKSSTLKHRANVQYVAENGSTLKEKSKAVGKKCRFPEDLD-TNKKVKKSTPRTKTS 624
 Newt KIVKSSTLKHRTRVVHVVENSSQTLKDNK-ISKKCRFSEDLD-VNKKAKRPVPTKKTP 614
 Zebrafish KIVKNSASSKSRPQVFEHVSETVRDKHRTGSRRTQVDEVHLLHKSSKKASAKTKRIP 617
 : : * : : : * : : * : : * : : : *

Human G--GPEAGVPGRPAG-GGHRAGSRAHGHR--EAVVAKPKHKRTDYRRW-KSSAEISYEE 689
 Mouse G--LQDAGLPGRALGTGGHRAGSRAHAGR--EPVVAKPCHKRTDYRRW-KSSAEVS YEE 668
 Chicken GGLQPESGLQGRPTG--LHKS VVRSHGHR--E VVVAKPCHKRADYRRW-KSSAEISYEE 676
 Xenopus HPNFEPAVVGRNPVA---VRSGIKSHGQPK--DVVLAKPKHKRGDYRRW-KSSAEISYEE 678
 Newt HLQVENGLPSSRLX---TTLRHPRHGHR--EGVLTMPKYKRGDYRRG-KSPAEMS YEE 668
 Zebrafish ASIPEGRILERHTSSSGARSSAQRRHGHHRHHAVALAKPKYKRNDFHRRPGLHEIFYEE 677
 . : : * : : : * : : * : : * : : * : : *

Human ALRRARR-GRRENVGLYPAPVPLPYASPYAYVASDSEYSAECESLFHSTVVDTSEDEQSN 748
 Mouse ALRRARR-ARREHGAAYRVAVALPYASPYAYVPSDSEYSAECESLFHSTVVDTSEDEQSN 727
 Chicken ALRRARR-NRRDGVGVY-AQVPLPVVSPYAYVASDSEYSAECESLFHSTVVDTSEDEQSN 734
 Xenopus ALRRARRRAQREMMGVY-AQVFPYPYASPYAYIASDSEYSAECESLFHSTVVDTSEDEQSN 737
 Newt ALRRARRRPGMEPVGVFAKVQFPYPYASPYAYIASDSEYSAECESLFHSTVVDTSEDEKSN 728
 Zebrafish AYRRARRRQKREMLSHMYLPSNAHYTSPYAYVGSDEYSAECASLFHSTILDSEDEKSN 737
 * ** : : * * : : * : : * : : * : : *

Human YTTNCFGDSESSVSEGEFVGESTTTSDSESGGLIWSQFVQTLPIQTVTAPDLHNHPAKT 808
 Mouse YTTNCFGDSESSVSEGEFVGESTTTSDSESGGLIWSQFVQTLPIQTVTAPDLHTRPKT 787
 Chicken YTTNCFGDSESSLSEVEFVGESTTTSDSESGGLIWSQFVQTLPIQTVTAPDLHENA AKA 794
 Xenopus YTTNCFGDSESSLSEVEFVGESTTTSDSESGGLIWSQFVHTLPMQATATAELQT-TAKA 796
 Newt YTTNCFGDSESSLSEVEFVGESTTTSDSESGGLIWSQFIQTLPMQTTTATELQK-TAKA 787
 Zebrafish YTTNCFGDSESSASEADYVAESSTSSDSEGSAGVNRQISQAG---SGSHGMTSAQAKA 793
 * : : * : : * : : * : : * : : * : : *

Human FVKIKASHNLKKKILRFRSGSLKLMTTV 836
 Mouse FVKIKASHNLKKKILRFRSGSLKLMTTV 815
 Chicken FVKIKASHNLKKKILRFRSGSLKLMTTV 822
 Xenopus FVKIKASHNLKKKILRFRSGSLKLMTTV 824
 Newt FVKIKASHNLKKKILRFRSGSLKLMTTV 815
 Zebrafish FVKIKASHNLKKKILRFRSGSLKLMTTV 821
 * : : * : : * : : * : : * : : *

[Figure 11](#) shows the multiple sequence alignment for both T β 4 and T β 10/15. Newt T β 4 is compared to the same protein from human, mouse, chicken, frog, and fish and is highly conserved (~80% at the protein level) among vertebrates throughout the coding region ([Fig. 11A](#)). Newt T β 10/15 is compared to human and chicken T β 15 and human and mouse T β 10 to emphasize its similarity to both proteins, as well as the differences T β 10/15 exhibits between the two ([Fig. 11B](#)). Newt T β 10/15 is highly conserved (~78% at the protein level) among vertebrates throughout the coding region.

3.5 – qPCR Analysis of Candidate Genes in Regenerating Limbs, Tail, and During A2 Myoblast Differentiation

3.5.1 – Genes of the Wnt Signalling Pathway

Quantitative PCR was performed to verify the differential regulation of *β -catenin*, *wntless*, and *dapper* and identify their expression patterns throughout regeneration in the limb, tail, and during A2 differentiation ([Fig. 12](#)). For limb regeneration, amputations were performed at the wrist and, at various time points following the initial amputation (e.g., R1, R3, R5, R7, R15, and R21), regenerating tissue was collected and mRNA was isolated. Quantitative PCR was conducted with gene-specific primers for *β -catenin*, *wntless*, and *dapper* with at least three separate samples per time point. The data were then pooled to provide the profile shown in [Fig. 12A](#). During limb regeneration, *dapper* and *wntless* trend towards increased upregulation, while *β -catenin* upregulation peaks early (~R3) and trends downward following dedifferentiation ([Fig. 12A](#)). The data from R3 validate the differential regulation indicated by the RDA for *β -catenin* and *wntless* (i.e., upregulation) but do not validate the differential regulation of *dapper* (which was

Figure 11. Multiple sequence alignment of *N. viridescens* thymosin- β protein sequences with that of human, mouse, chicken, frog, and/or fish. (A) Newt T β 4 (highlighted in yellow) is highly conserved (~80% at the protein level) among vertebrates. (B) Newt T β 10/15 (highlighted in yellow) is compared to human and chicken T β 15 and human and mouse T β 10. This comparison emphasizes the similarities and differences newt T β 10/15 has with each protein. Newt T β 10/15 is highly conserved among vertebrates (~78% at the protein level). Alignment performed with ClustalW2 multiple sequence alignment tool (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). [Legend: “*” = all residues in column are identical; “:” = conserved substitutions are observed; “.” = semi-conserved substitutions are observed; red = small + hydrophobic (including aromatic –Y); blue = acidic; magenta = basic – H; green = hydroxyl + sulfhydryl + amine + G; grey = other]

A

Human	MSDKPDMAEIEKFDKSKLKKTTETQEKNPSPKETIEQEKQAGES--	44
Chicken	MSDKPDMAEIEKFDKSKLKKTTETQEKNPSPKETIEQEKQAGESK-	45
Mouse	MSDKPDMAEIEKFDKSKLKKTTETQEKNPSPKETIEQEKQAGES--	44
Xenopus	MSDKPDMAEIEKFDKAKLKKTTETQEKNPSPKETIEQEKQSTES--	44
Newt	MSDKPDMGEVENFDRALKKTTETQEKNPSPKETIEQEKKNITES--	44
Zebrafish	MADKPNMTEITSFDKTKLRKTTETQEKNPSPKETIEQERQ-GESTP	45

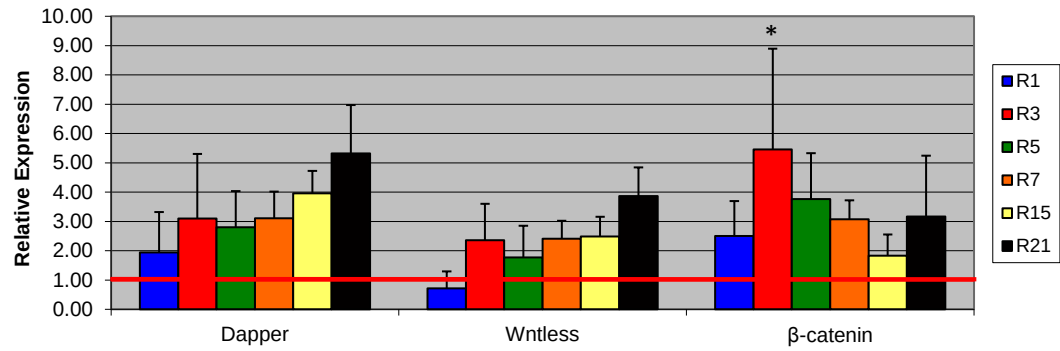
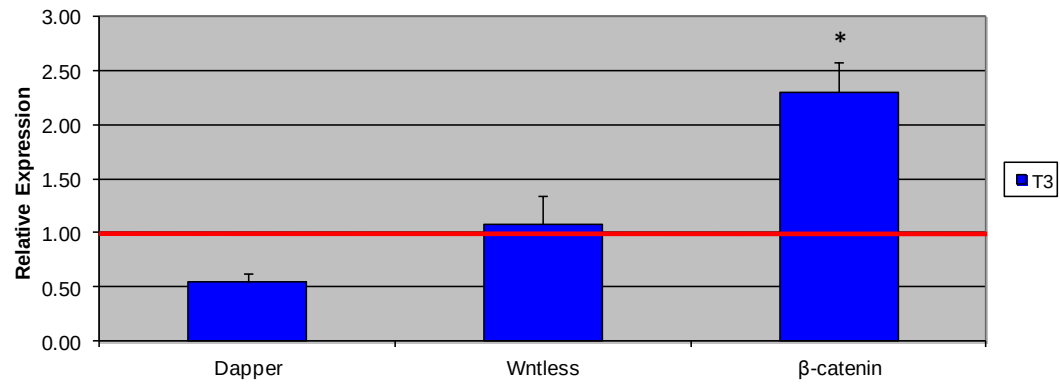
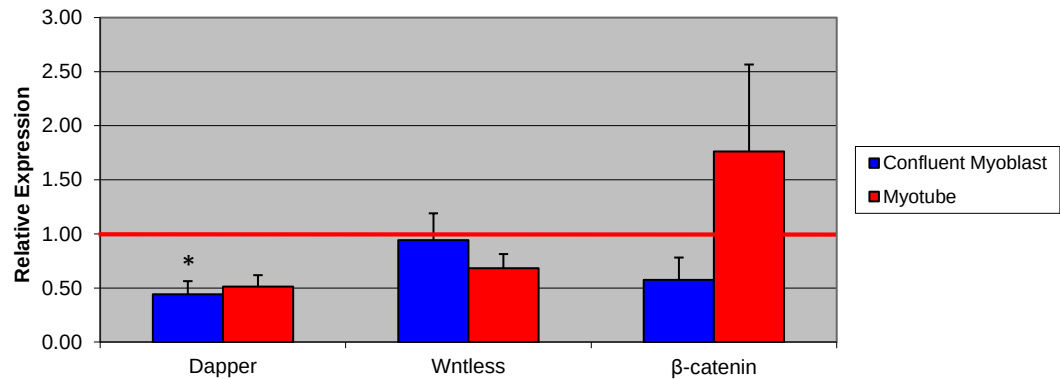
::*:* * : .*:*:**:*:*:*:*:*:*:*:*:*:*:*:*:*:* *

B

Chicken (TB15)	MCDKPDLSEVEKFDKSKLKKTNTEKNTLPSKETIEQEKQCVKSS	45
Human (TB15)	MSDKPDLSEVEKFDRSKLKKTNTEKNTLPSKETIQEKECVQTS	45
Newt (TB10/15)	MSDKPDMSEVENFDIQMLKKTTETQEKNTLPSKETIEQEKQACKKS	45
Human (TB10)	-----MGEIASFDKAKLKKTTETQEKNTLPTKETIEQEKRSSEIS-	38
Mouse (TB10)	MADKPDMEIASFDKAKLKKTTETQEKNTLPTKETIEQEKRSSEIS-	44

:.**: .** *****.*:*:*:*:*:*:*:*:*:*.*

Figure 12. Verification and analysis of the differential expression of three wnt pathway genes identified in the RDA. (A) Relative expression of *dapper*, *wntless*, and β -*catenin* throughout the time course of limb regeneration in comparison to the intact limb (R0; red line). Both *dapper* and *wntless* show a trend of increasing upregulation as limb regeneration proceeds while β -*catenin* peaks early ($\sim$ R3) and trends downward as regeneration continues. The R3 data validate the differential regulation indicated by the RDA for β -*catenin* and *wntless* (i.e., upregulation) but do not validate the differential regulation of *dapper* (identified as downregulated by the RDA). **(B)** Relative expression of *dapper*, *wntless*, and β -*catenin* at three days post-amputation (T3) in the tail in comparison to the intact tail (T0; red line). β -*catenin* is upregulated, indicating a similar trend to the limb for differential regulation of β -*catenin* during tail regeneration. *Dapper* appears to be downregulated (although not significant) and *wntless* shows no significant change in expression in the T3 tail regenerate. **(C)** Relative expression of *dapper*, *wntless*, and β -*catenin* during A2 myoblast differentiation in comparison to sub-confluent myoblasts (SCMB; red line). β -*catenin* trends towards upregulation in the myotube culture while expression of *dapper* and *wntless* are trending down as differentiation proceeds. Limb and tail cDNA samples were pooled from ≥ 10 newts while myoblast/myotube cDNA samples were pooled from $\geq 2 \times 10^6$ plates. qPCR was performed in triplicate with $n \geq 3$ for **A**; statistical analysis for **A** and **C**: one-way ANOVA; **B**: Student's T-Test (* $p < 0.05$ for technical and biological replicates; error bars represent SEM). Gene expression was normalized to *GAPDH* and samples compared to their respective control (red line; **A**: R0, **B**: T0, **C**: sub-confluent myoblasts).

A**Average Expression of Wnt Signalling Genes in Regenerating Newt Limb****B****Expression of Wnt Signalling Genes in Regenerating Newt Tail****C****Average Expression of Wnt Signalling Genes During A2 Differentiation**

identified as downregulated by the RDA). Potential reasons for this discrepancy will be addressed in the discussion (see [Sections 4.2.1](#) and [4.3.4](#)).

For tail regeneration, the distal 1.5cm portion of the tail was removed and a 1mm piece of the stump was amputated for the intact tail sample (T0). Regenerating tissue was collected at three days (T3) following the initial amputation and mRNA was isolated. Once again, qPCR was conducted with gene-specific primers for *β -catenin*, *wntless*, and *dapper* to compare their expression at the T0 and T3 time points. Due to the difficulty obtaining sufficient mRNA from tail samples, this experiment was completed only once (*i.e.*, tissue from multiple newts was combined into one sample, with qPCR performed in at least three technical replicates). During early tail regeneration, *β -catenin* is upregulated and *dapper*, while not significant, trends towards downregulation ([Fig. 12B](#)). This differential regulation of *β -catenin* during early tail regeneration indicates a similar trend to that seen in the limb. *Wntless* shows no significant change in expression during the early stage of tail regeneration ([Fig. 12B](#)).

Finally, for A2 differentiation, sub-confluent and confluent myoblasts (SCMB, and CMB, respectively) were collected from 10cm plates. Serum was dropped to induce differentiation and, four days later, myotube (MT) cultures were collected. Messenger RNA was isolated from the samples and qPCR was once again performed, in triplicate, with gene-specific primers for *β -catenin*, *wntless*, and *dapper*. During A2 myoblast differentiation, although not significant, *β -catenin* appears to be upregulated in the MT culture ([Fig. 12C](#)). The trend for *dapper* indicates downregulation throughout differentiation (only statistically significant in the CMB cultures compared to the SCMB

control), while *wntless* shows no significant change from control levels throughout differentiation ([Fig. 12C](#)).

3.5.2 – *Thymosin-β* Genes

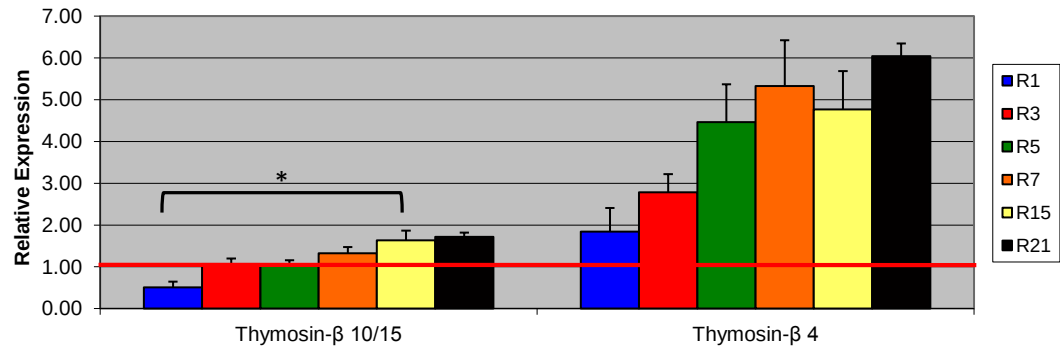
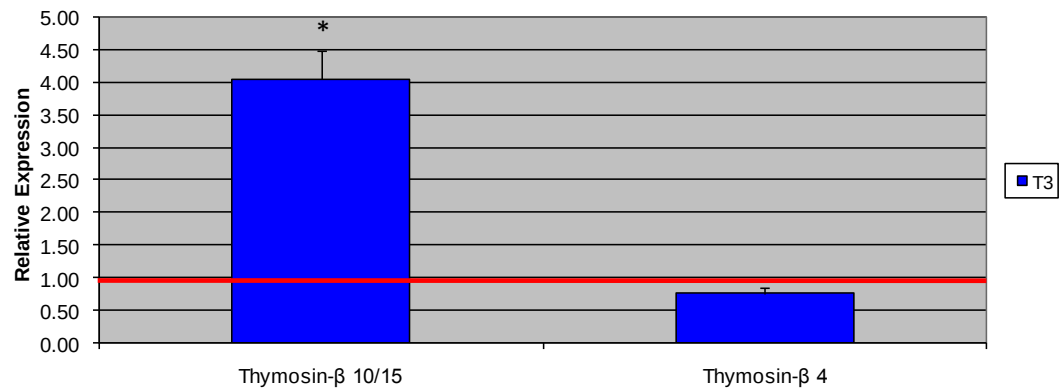
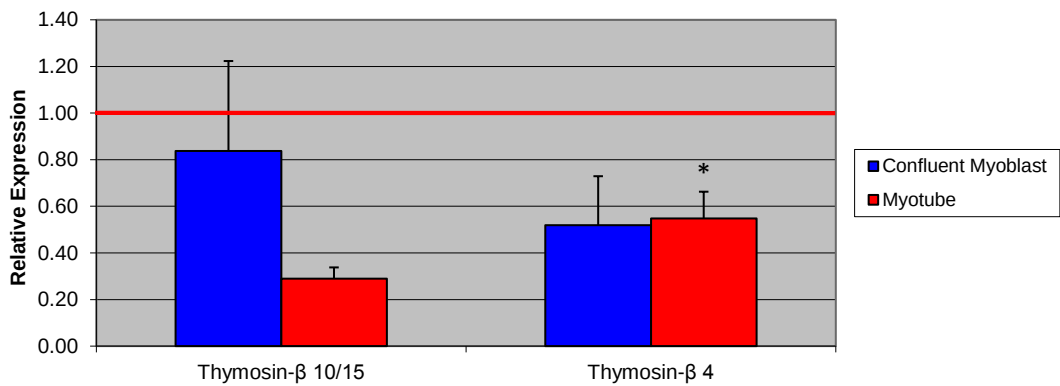
Quantitative PCR was also conducted for *Tβ4* and *Tβ10/15* to verify their differential regulation and identify their expression patterns throughout regeneration in the limb/tail and during A2 differentiation ([Fig. 13](#)). Messenger RNA was isolated and cDNA synthesized from the same time points as described above for the genes of the wnt signalling pathway (see [Section 3.5.1](#)). Primers for amplification of *Tβ4* and *Tβ10/15* were synthesized from sequence in the 3'UTR (untranslated region) of the genes in order to obtain gene-specific amplification.

During limb regeneration, *Tβ4* expression steadily increases ([Fig. 13A](#)). *Tβ10/15*, however, is only significantly upregulated at R15 compared to R1 ([Fig. 13A](#)). The data from R3 do not validate the differential regulation indicated by the RDA/microarray for *Tβ10/15* (i.e., upregulation). In contrast to the limb samples, *Tβ10/15* is upregulated at T3 in the early tail regenerate while *Tβ4* shows no change ([Fig. 13B](#)). This indicates a potentially different role for these genes during tail regeneration compared to the limb. During A2 myoblast differentiation, *Tβ10/15* appears to be downregulated as differentiation proceeds (although not reaching significance), while *Tβ4* is downregulated in the MT cultures ([Fig. 13C](#)).

3.6 – IHC Analysis of Candidate Genes in Regenerating Limbs

In order to further characterize the expression of the wnt signalling pathway genes and *Tβ4*, we performed IHC to visualize where the proteins were being expressed in

Figure 13. Verification and analysis of the differential expression of two *thymosin-β* genes identified in the RDA and RACE. (A) Relative expression of *Tβ10/15* and *Tβ4* throughout the time course of limb regeneration in comparison to the intact limb (R0; red line). *Tβ4* trends towards increasing upregulation throughout the time course of limb regeneration. *Tβ10/15* expression, however, only shows an increase at R15 compared to R1. The R3 data do not validate the differential regulation indicated by the RDA for *Tβ10/15* (i.e., upregulation). **(B)** Relative expression of *Tβ10/15* and *Tβ4* at three days post-amputation (T3) in the tail in comparison to the intact tail (T0; red line). In contrast to the limb regeneration samples, *Tβ10/15* is upregulated at T3 while *Tβ4* appears unchanged. **(C)** Relative expression of *Tβ10/15* and *Tβ4* during A2 myoblast differentiation in comparison to sub-confluent myoblasts (SCMB; red line). Both genes appear to be downregulated during differentiation, although the results are only significant in MT cultures for *Tβ4*. Limb and tail cDNA samples were pooled from ≥ 10 newts while myoblast/myotube cDNA samples were pooled from $\geq 2 \times 10^6$ cells. qPCR was performed in triplicate with $n \geq 3$ for **A**; statistical analysis for **A** and **C**: one-way ANOVA; **B**: Student's T-Test (* $p < 0.05$ for technical and biological replicates; error bars represent SEM). Induction was normalized to *GAPDH* and samples compared to their respective control unless otherwise indicated (red line; **A**: R0, **B**: T0, **C**: sub-confluent myoblasts).

A**Average Expression of Thymosin- β Genes in Regenerating Newt Limb****B****Expression of Thymosin- β Genes in Regenerating Newt Tail****C****Average Expression of Thymosin- β Genes During A2 Differentiation**

regenerating limbs. To do this, newt forelimbs were amputated at the mid-radius/ulna to induce a regeneration response. At various time points post-amputation (R3, R7, R15, and R21), the regenerating limbs were amputated at the mid-humerus level and subjected to a decalcification protocol before being embedded for sectioning (intact limbs were also amputated at the mid-humerus level for the R0 time point). Limb sections were subjected to IHC using anti- β -catenin and anti-T β 4 antibodies. First, however, in order to better illustrate the overall morphology and anatomy of longitudinal limb sections, haematoxylin and eosin (H & E) staining was performed ([Fig. 14](#)). The amputated bone (b; radius/ulna) is clearly visible in the regenerating limb sections ([Fig. 14B-D](#)), as is the blastema (bl) in the mid-blastema and late blastema/early redifferentiation stages (R15 and R21, respectively; [Fig. 14C and D](#); note: the limb sections here have been defined differently than the standard time points to reflect the fact that they don't appear to be as advanced as the standard timing; *i.e.*, R15 in [Fig. 14C](#) appears to be more mid-blastema than late and R21 in [Fig. 14D](#) appears more late blastema than early redifferentiation).

The H & E images help to orient and localize the signals obtained by IHC ([Figs. 15 and 16](#)) in the context of the gross morphology of the regenerating limb. Due to the high conservation of newt β -catenin protein in relation to the human sequence, a human specific antibody was used on newt sections. A Western blot was also performed to verify the specificity of the β -catenin antibody ([Fig. 15A](#)). [Figure 15 C-G](#) shows the localization of β -catenin protein in intact and regenerating limbs. The levels of β -catenin are highest at R3 in the area around and just proximal to the amputation plane (yellow line; [Fig. 15D](#)). Blastema cells also appear to be prominently labelled. Levels of β -catenin slowly decrease as regeneration proceeds and the protein becomes further

Figure 14. Haematoxylin and eosin staining of intact and regenerating newt limbs at R7, R15, and R21. (A) Composite image of a longitudinal section through an intact (R0) newt limb in the zeugopod. The section is taken above the plane of the bone. Muscle (m) is most prominent while mucus glands (mg) can be seen below the thin epithelium (e). **(B)** Composite image of a longitudinal section through an early blastema (R7) limb regenerate. A large bone fragment (b; radius/ulna) is visible just proximal to the amputation plane. **(C)** Composite image of a longitudinal section through a mid-blastema (R15) limb regenerate. The blastema (bl) is clearly evident at this stage. **(D)** Composite image of a longitudinal section through an R21 limb regenerate. In this stage, the blastema is fully established and redifferentiation of blastema cells will begin to occur in the proximal portion of the regenerate. The dashed line indicates the approximate level of amputation.

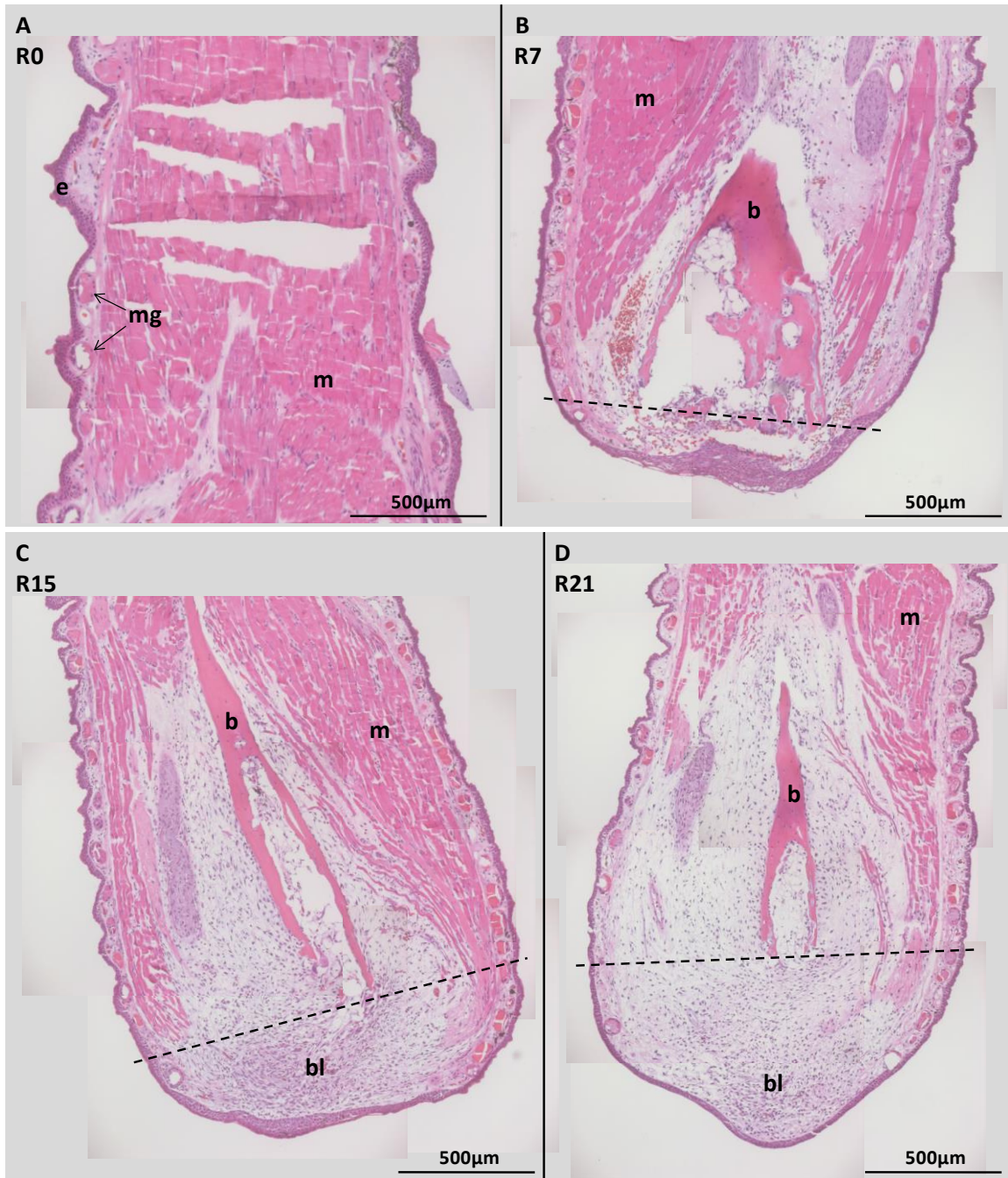


Figure 15. IHC for β -catenin in the intact and regenerating newt limb at various stages in the regeneration process. (A) Western blot showing specificity of the β -catenin antibody used for IHC in R0 and R7 protein isolated from newt limbs at the respective time point. Expected size of β -catenin is ~90kDa. (B) No primary control indicates background fluorescence (red) in mucus glands (mg) and artefactual folds in the section. (C) Relatively little β -catenin is seen at R0. (D) There is a marked increase in protein levels just proximal to and at the amputation plane at R3 (peak dedifferentiation). (E, F) Composite images of R7 limb (early blastema; E) and R15 limb (mid-blastema; F) show specific β -catenin expression in the blastema (bl) and adjacent to the amputated bone (b). Inset E: Magnified view of an image from the R7 blastema to better show β -catenin within the blastema. (G) R21 shows β -catenin even more specific to the blastema. All sections are counterstained with DAPI to visualize nuclei with the distal part of the limb oriented to the bottom. The dashed line indicates the approximate level of amputation. (Legend: e = epithelium, mg = mucus gland; m = muscle; b = bone; bl = blastema)

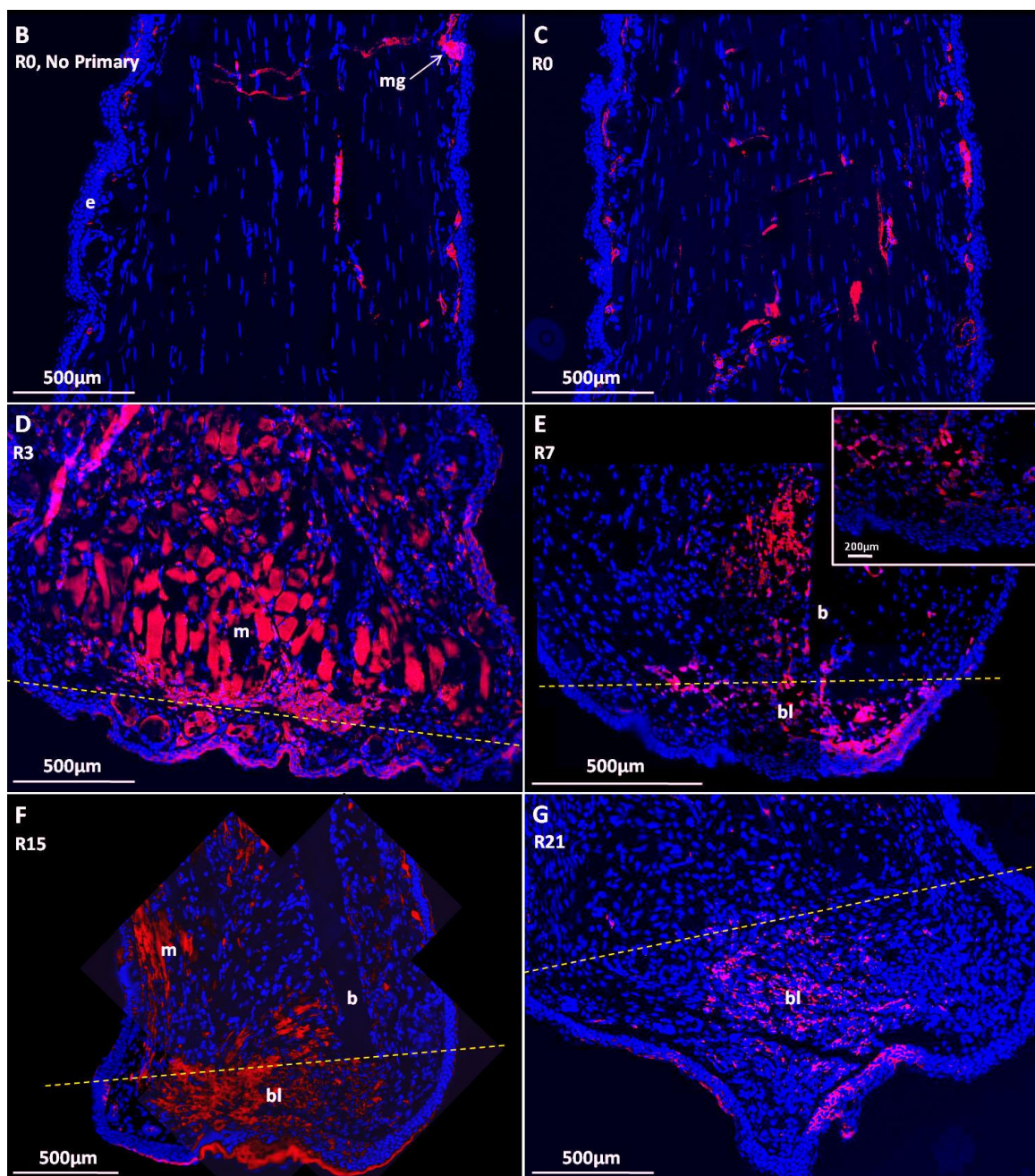
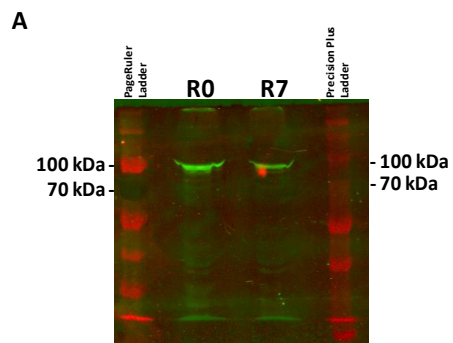
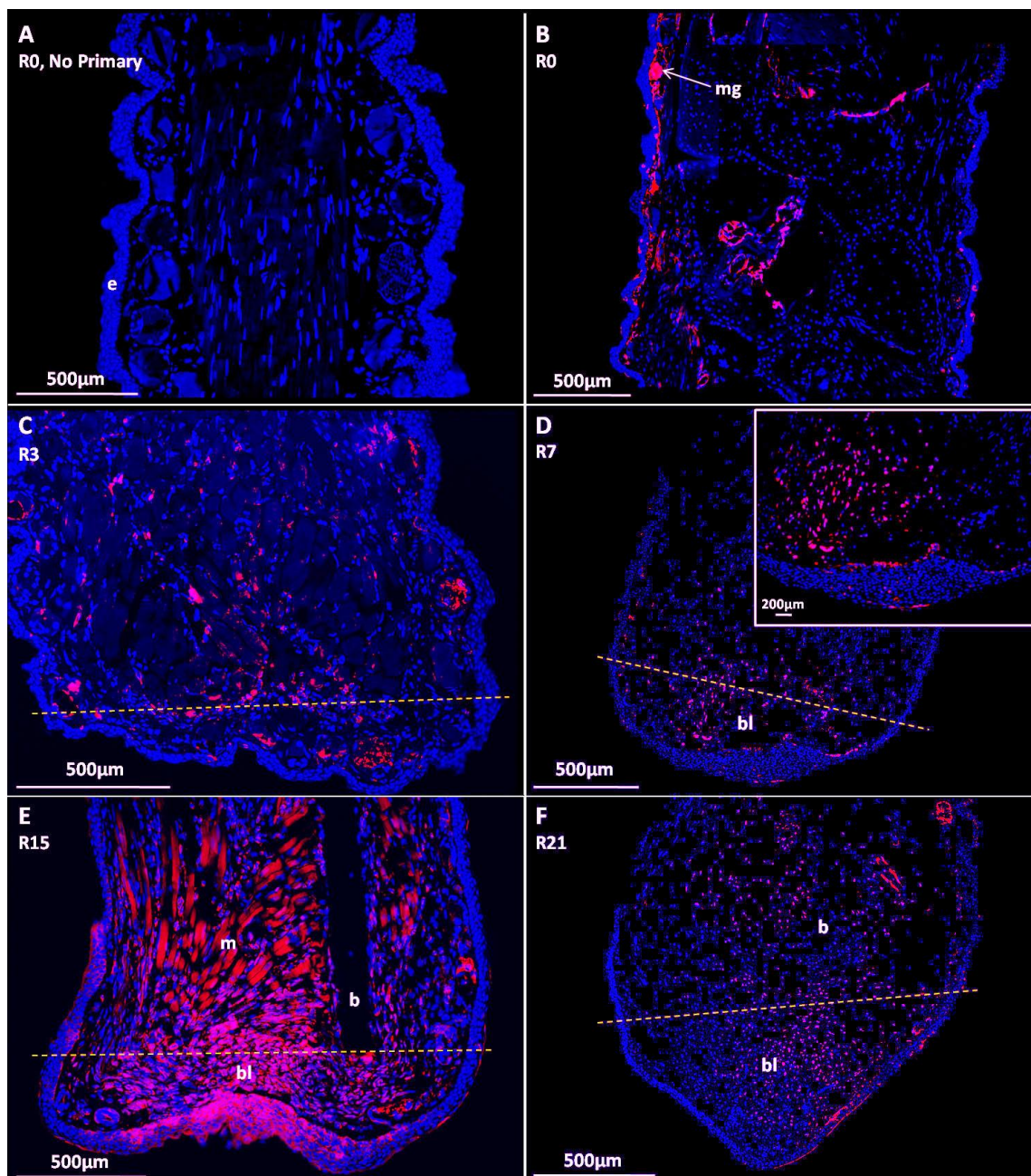


Figure 16. IHC for thymosin- β 4 in the intact and regenerating newt limb at various stages in the regeneration process. (A) No primary control indicates no background fluorescence. (B) Relatively little T β 4 is seen in the composite R0 image. (C) There is an increase in protein levels at the amputation plane in R3 (peak dedifferentiation). (D) Composite image of R7 limb (early blastema) shows T β 4 is specific to the blastema (bl). Inset: Magnified view of an image from the R7 blastema to better show T β 4 within the blastema. (E) Image of R15 limb (mid-blastema) shows T β 4 spreading proximally from the amputation plane to the muscle (m). (F) R21 shows T β 4 spread throughout the distal regenerating limb. All sections are counterstained with DAPI to visualize nuclei with the distal part of the limb oriented to the bottom. The dashed line indicates the approximate level of amputation. (Legend: e = epithelium, mg = mucus gland; m = muscle; b = bone; bl = blastema)



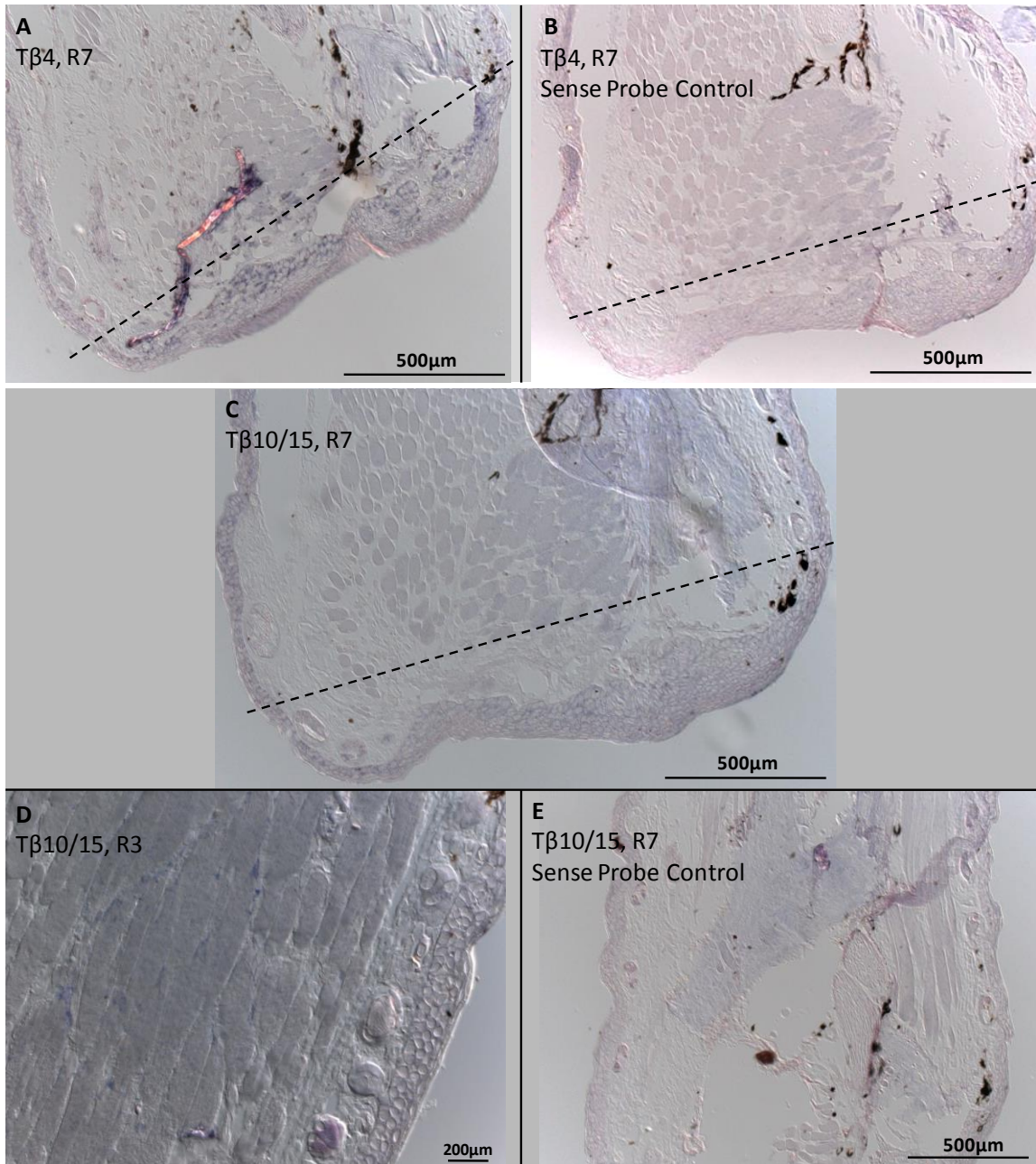
restricted to the area of the blastema ([Fig. 15E-G](#)).

[Figure 16](#) shows the localization of T β 4 protein in intact and regenerating limbs. The levels of T β 4 slowly increase throughout the regeneration time course ([Fig. 16C-F](#)). At R3, T β 4 expression is sporadic throughout the distal end of the regenerating limb ([Fig. 16C](#)). By R7, T β 4 expression is quite strong within the blastema ([Fig. 16D](#)). At R15, T β 4 expression is strong throughout the distal regenerating tip ([Fig. 16E](#)). Finally, R21 shares a similar profile to R15, with T β 4 expression being fairly ubiquitous throughout the distal part of the regenerating limb ([Fig. 16F](#)). A Western blot was unable to verify the specificity of the T β 4 antibody as the protein was too small for the available membranes (*i.e.*, <5kDa) and, given the small size and high homology between T β family members, we were unsure if the human-specific anti-T β 4 antibody would bind to T β 4 only.

3.7 – ISH Analysis of Candidate Genes in Regenerating Limbs

Because we were unable to verify the specificity of the T β 4 antibody used for IHC, and because there is a high degree of homology between T β 4 and T β 10/15, ISH was performed on regenerating limb sections to determine if there was a difference in the localization of *T β 4* and *T β 10/15* transcripts ([Fig. 17](#)). To do this, newt forelimbs were amputated at the mid-radius/ulna to induce a regeneration response. At various time points post-amputation (R3, R7, R15, and R21), the regenerating limbs were amputated at the mid-humerus level and subjected to an RNase-free decalcification protocol before being embedded for sectioning (intact limbs were also amputated at the mid-humerus level for the R0 time point). Limb sections were subjected to ISH with DIG-labelled probes conjugated to alkaline phosphatase (AP) for a colourimetric assay (*i.e.*, visible

Figure 17. ISH verifies specificity of IHC for thymosin- β 4 in the regenerating newt limb. (A) *T β 4* transcript is present (purple stain) in the blastema (bl) in the R7 limb and is seen in the apical epithelial cap (AEC). (B) Lack of staining with the *T β 4* sense probe control indicates specificity of the anti-sense probe in A. (C) *T β 10/15* transcript is not present in R7 limb blastema, within the limits of detection of the assay. (D) Anti-sense probe for *T β 10/15* shows a low level of signal in R3 muscle tissue (m), suggesting that the probe is specific. (E) Minimal background is seen in the R7 limb with the *T β 10/15* sense probe. Dashed line indicates approximate level of amputation. (Legend: e = epithelium, mg = mucus gland; m = muscle; b = bone; bl = blastema; AEC = apical epithelial cap)



purple staining). *Tβ4* transcript is visible (purple stain) in the early blastema in R7 limbs ([Fig. 17A](#)), whereas *Tβ10/15* is not ([Fig. 17C](#)), within the limits of detection of the assay. The *Tβ10/15* probe was specific as purple staining is evident in muscle tissue located more proximally in the limb ([Fig. 17D](#)). Sense-control probes for both transcripts showed minimal levels of background staining ([Fig. 17B and E](#)). These data indicate that the Tβ4 antibody staining seen in [Fig. 16](#) is most likely specific for the Tβ4 protein since the ISH and IHC showed similar profiles.

3.8 – Functional Analysis of Candidate Genes in C2C12 Myoblasts

We initially intended to perform the majority of our functional analyses on newt A2 myoblasts and differentiated myotubes. However, as these cells are extremely difficult to efficiently transfect, obtaining stable cell lines would be extremely time consuming. Thus, we decided to use mouse C2C12 cells. C2C12 cells are an immortalized myoblast cell line which are more amenable to transfection and undergo a differentiation process similar to A2s.

3.8.1 – Candidate Gene Expression During C2C12 Myoblast Differentiation

The first step in determining the functional role of the genes of the wnt signalling pathway and *Tβ4* in C2C12 cells was to determine the normal level of expression of these genes during myoblast differentiation. For these studies, C2C12 cells were grown to confluency and the serum in the medium was dropped to induce cell cycle exit and differentiation into multinucleated myotubes. Cells were sampled at various stages: sub-

confluent myoblasts (SCMB), confluent myoblasts (CMB), and 24, 48, and 96 hours after dropping the serum to induce myotube differentiation (MT). [Figure 18A-D](#) shows the relative expression by qPCR of each gene (*β-catenin*, *wntless*, *dapper*, and *Tβ4*, respectively) in CMB and 24, 48, and 96 hour MT cultures in comparison to SCMB cultures. *β-catenin* is upregulated at the 24h MT stage compared to both the SCMB control and CMB cultures ([Fig. 18A](#)). *Wntless* is upregulated throughout differentiation, peaking in the CMBs and declining and stabilizing in the MT stages ([Fig. 18B](#)). *Dapper* expression also appears to be upregulated throughout differentiation, although there is no statistical significance ([Fig. 18C](#)). Finally, *Tβ4* shows no significant change from control levels throughout C2C12 myotube differentiation ([Fig. 18D](#)).

3.8.2 – Candidate Gene Overexpression in C2C12 Myoblasts

C2C12 myoblasts were transfected with pCI-Neo vector containing one of the four newt genes (*β-catenin*, *wntless*, *dapper*, and *Tβ4*) and selected with G418 until stable lines were developed. For each of the cell lines, qPCR was conducted with primers designed to detect both the mouse and newt versions of the genes to determine if they were indeed being overexpressed, compared to the pCI (empty vector) control cell line ([Fig. 19](#)). In addition, qPCR was conducted on each cell line with primers for the other three genes to determine if overexpression of one gene caused expression changes in the others. Only the newt *dapper* overexpressing C2C12 myoblast line showed significant upregulation of the transfected gene ([Fig. 19B](#)). The newt *Tβ4* overexpressing cell line did indicate an increase in total *Tβ4*, although this was not significant ([Fig. 19C](#)). Unfortunately, the newt *β-catenin* and *wntless* overexpressing cell lines did not indicate an upregulation of total levels of these genes, respectively ([Fig 19A and D](#)).

Figure 18. Expression of β -catenin, *wntless*, *dapper*, and *thymosin- β 4* during C2C12 myoblast differentiation. (A) β -catenin expression peaks at 24h after dropping the serum and inducing differentiation and appears to decline as cells become further differentiated. (B) *Wntless* expression peaks in confluent myoblasts and drops and stabilizes in the differentiating myotubes. (C) *Dapper* expression appears to be upregulated throughout differentiation (although not significant). A-C all indicate higher expression throughout differentiation compared to sub-confluent myoblast controls. (D) *T β 4* shows no change in expression from sub-confluent myoblast controls throughout differentiation. qPCR was performed in quadruplicate with n=3 (*i.e.*, four technical replicates on each of three biological replicates; one-way ANOVA, *p<0.05; error bars represent SEM). Gene expression was normalized to both *GAPDH* and *HPRT* and samples were compared to sub-confluent myoblast controls (SCMB, red line), unless otherwise indicated.

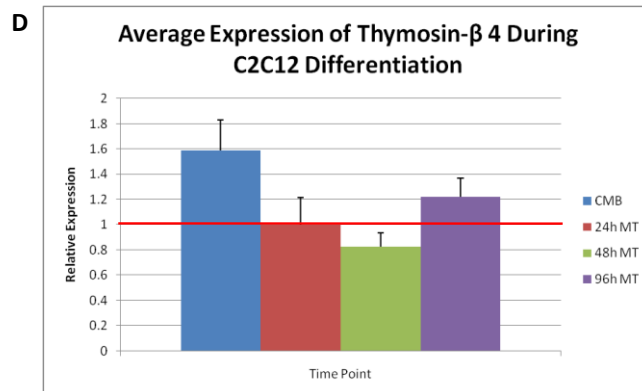
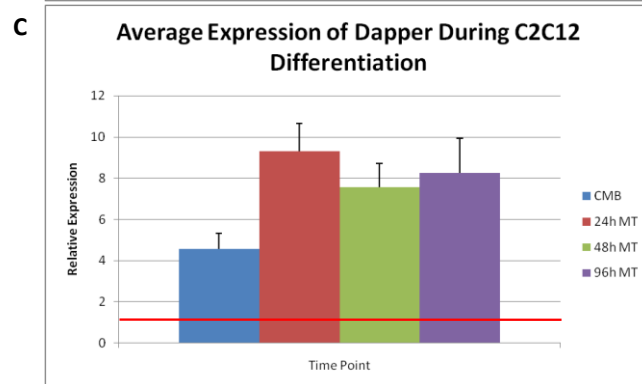
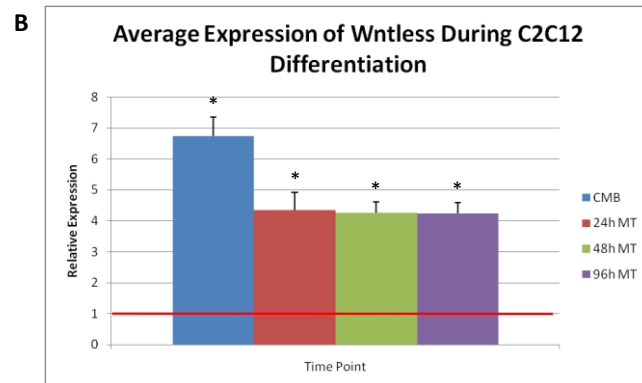
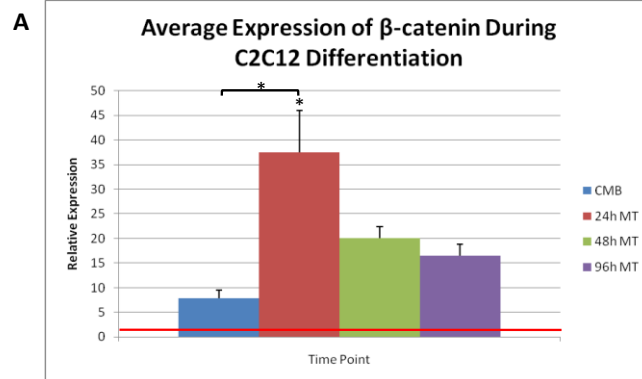
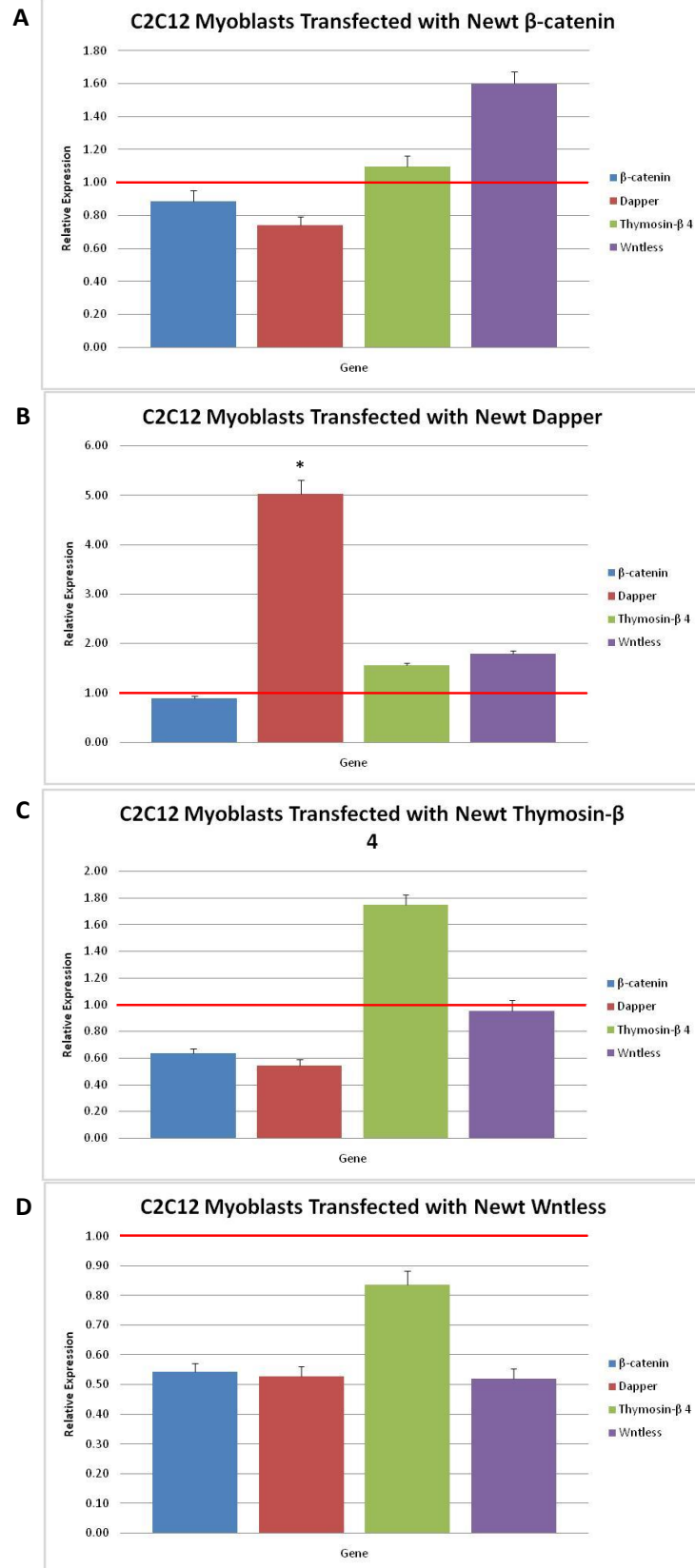


Figure 19. Expression of β -catenin, *wntless*, *dapper*, and *thymosin- β 4* in C2C12 myoblasts transfected with newt genes. (A) Newt β -catenin expressing C2C12 myoblasts show no significant change in the expression of any of the genes tested. (B) Newt *dapper* expressing C2C12 myoblasts show overexpression of total *dapper*. (C) Newt *T β 4* expressing C2C12 myoblasts indicate overexpression of total *T β 4*, although this is not significant. (D) Newt *wntless* expressing C2C12 myoblasts also show no change in the expression of any of the genes tested. qPCR was performed in quadruplicate with n=3 (*i.e.*, four technical replicates on each of three biological replicates; one-way ANOVA, *p<0.05; error bars represent SEM). Gene expression was normalized to both *GAPDH* and *HPRT* and samples were compared to pCI (empty vector) control (red line). Primers were designed to recognize both mouse and newt forms of the respective genes, thus “total” refers to the levels of both endogenous and exogenous transcripts.



3.8.3 – Candidate Gene Effects on Proliferation of C2C12 Myoblasts

In order to examine effects on the proliferation and survival of the C2C12 myoblasts transfected with the newt genes, a proliferation assay was performed. Approximately 2×10^4 cells were plated on either 35mm or 10cm plates and were cultured normally before harvesting at 24, 48, and 72 hours. Cells were counted and stained with a viability marker (trypan blue) to identify dead cells ([Fig. 20](#)). C2C12 myoblasts expressing newt *β -catenin* and *wntless* showed the greatest changes in cell counts compared to pCI (empty vector) control cells, with *β -catenin*-expressing cells growing faster and *wntless*-expressing cells growing slower than controls ([Fig. 20A](#)). Expression of newt *dapper* or *T β 4* had no effect on the proliferation rate of C2C12 myoblasts over three days. Quantitative PCR analysis for mouse *cyclinD1* showed that C2C12 myoblasts expressing newt *β -catenin* had increased *cyclinD1* transcript levels, correlating with the increased proliferation rate ([Fig. 20B](#)). When a live/dead assay was performed (*i.e.*, trypan blue staining), none of the newt genes caused significant cell death upon expression in C2C12 myoblasts over the three day time course ([Fig. 20C](#)).

3.8.4 – Candidate Gene Effects on Differentiation of C2C12 Myoblasts

We performed a differentiation assay to determine if any of the overexpressed newt genes would affect the proper differentiation of C2C12 myoblasts. We reasoned that any gene that prevents proper differentiation may play a role in dedifferentiation by helping cells to remain in and/or revert to a less differentiated state. [Figure 21](#) shows representative fluorescent micrographs of 96 hour myotube (MT) cultures for each C2C12 myoblast line from an IHC for myosin heavy chain (MHC, a mature muscle marker; red stain). Normal myotube formation is evident in the pCI control ([Fig. 21A](#)).

Figure 20. Newt β -catenin and wntless expression affect proliferation of C2C12 myoblasts. (A) Ratio of number of cells compared to pCI (empty vector) control shows expression of newt β -catenin increases proliferation of C2C12 myoblasts over three days in culture. Conversely, expression of newt wntless decreases the proliferation of C2C12 myoblasts over three days in culture. Expression of newt dapper or $T\beta 4$ has no effect on the proliferation of C2C12 myoblasts. Error bars represent SEM and are only shown for β -catenin and wntless for simplification. ($n \geq 3$; Student's T-Test, $*p < 0.05$) (B) Mouse *cyclinD1* is upregulated in newt β -catenin transfected C2C12 myoblasts while its levels remain unchanged in C2C12 myoblasts expressing newt dapper, wntless, and $T\beta 4$. qPCR was performed in quadruplicate with $n=3$ (i.e., four technical replicates on each of three biological replicates; Student's T-Test, $*p < 0.05$; error bars represent SEM). Gene expression was normalized to both *GAPDH* and *HPRT* and samples were compared to pCI (empty vector) control (red line). (C) Live/dead assay with trypan blue solution indicates minimal cell death during the three day proliferation assay in each C2C12 cell line transfected with a newt gene.

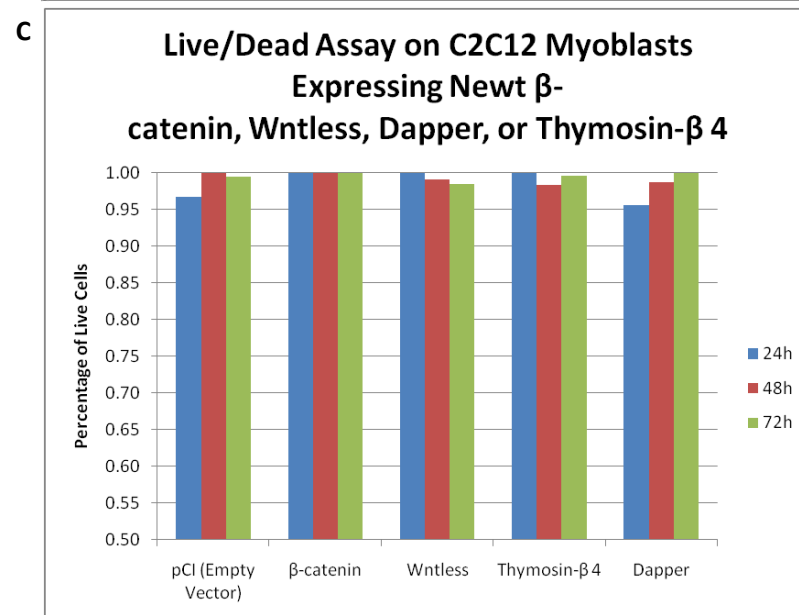
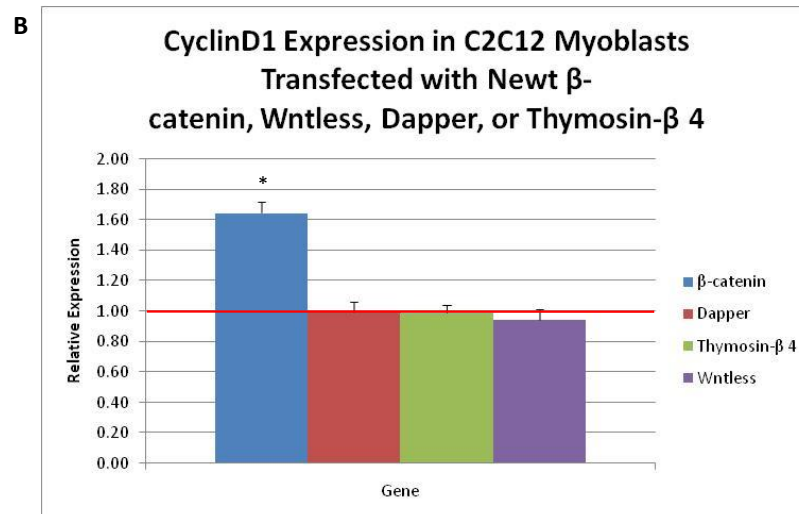
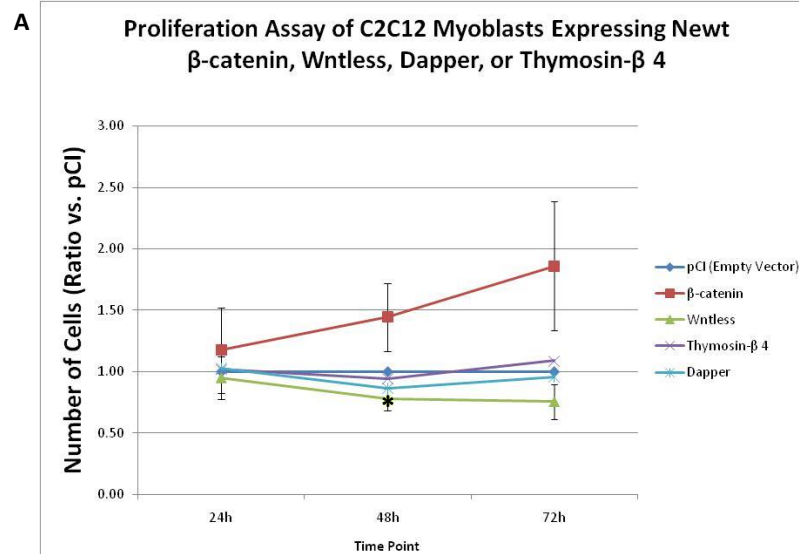
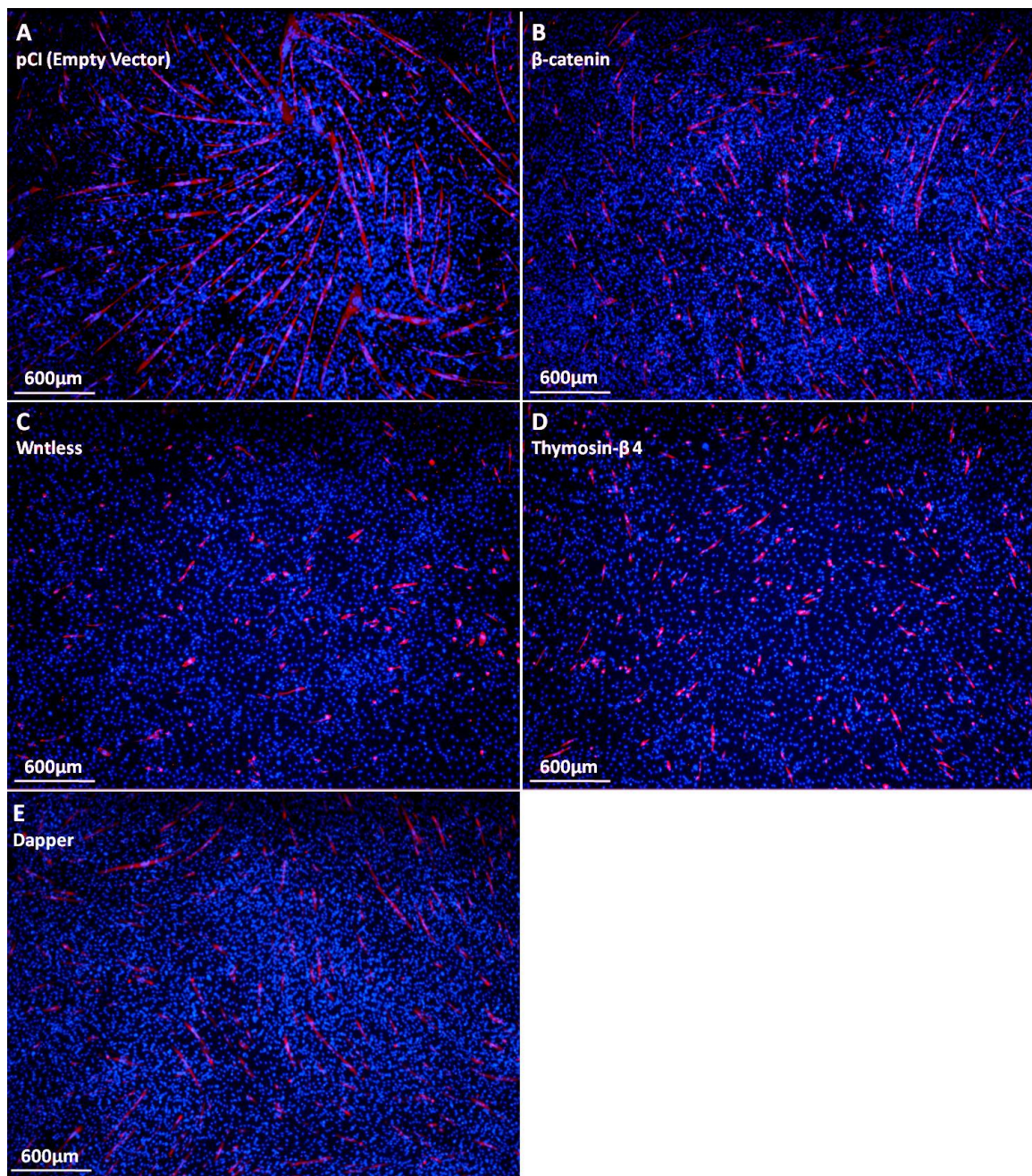


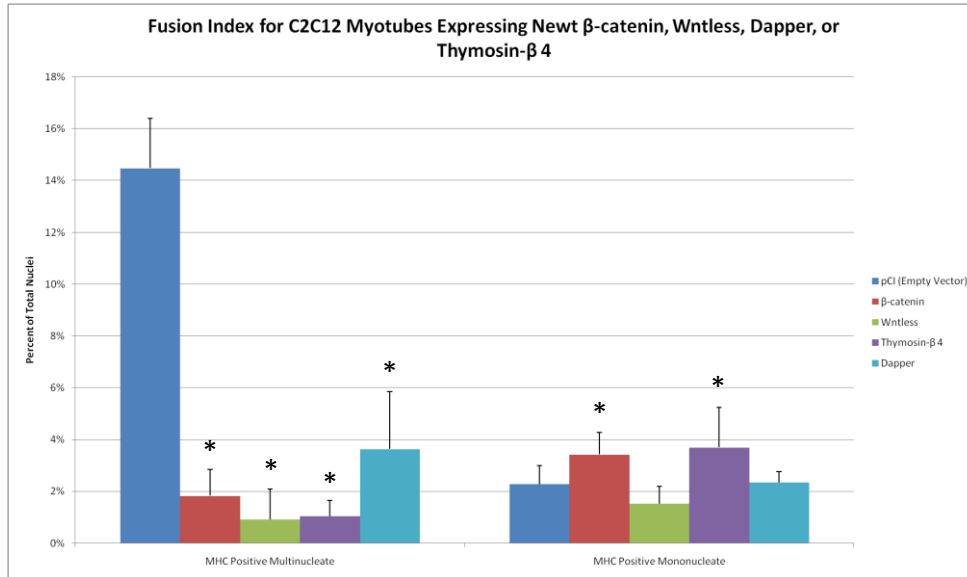
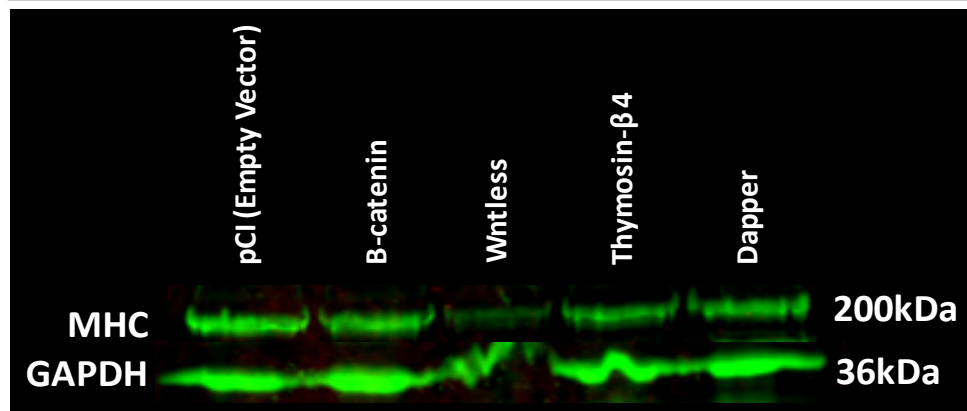
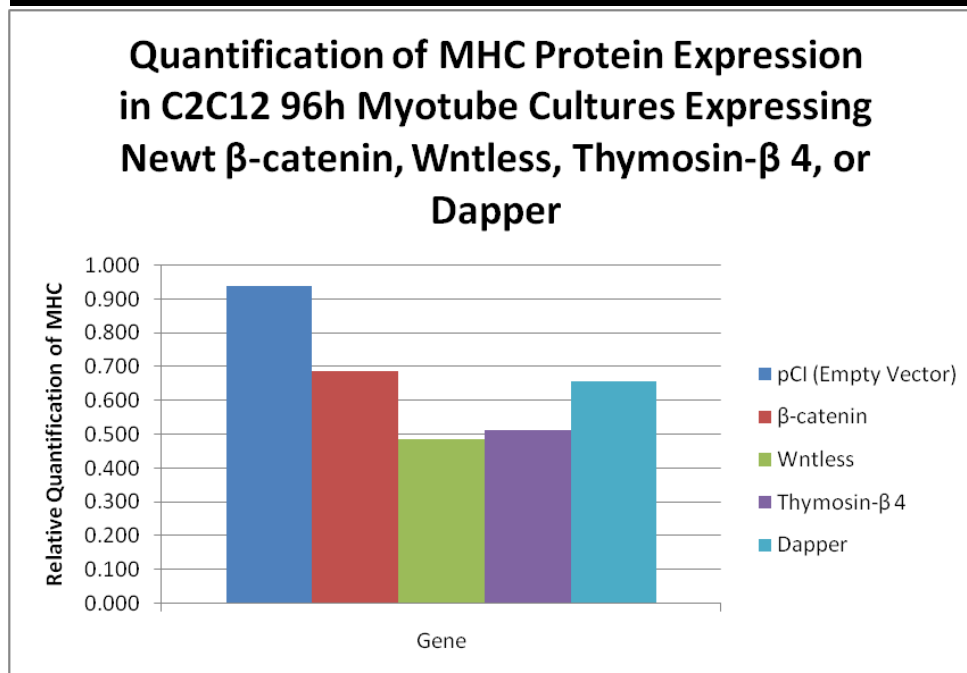
Figure 21. Expression of newt β -catenin, *wntless*, *thymosin- β 4*, and *dapper* inhibit proper differentiation of C2C12 myoblasts. (A-E) IHC for myosin heavy chain (MHC; red) during differentiation of C2C12 myoblasts expressing the control pCI vector or one of the newt genes. (A) pCI (empty vector) control shows normal differentiation of C2C12 myoblasts into myotubes at 96 hours. Expression of β -catenin, *wntless*, *T β 4*, and *dapper* (B-E, respectively) inhibit proper myotube formation, indicated by smaller myotubes (*i.e.*, fewer nuclei per myotube) and more MHC positive mononucleated cells at 96 hours of differentiation.



Expression of newt *β -catenin* ([Fig. 21B](#)), *wntless* ([Fig. 21C](#)), *T β 4* ([Fig. 21D](#)) and *dapper* ([Fig. 21E](#)) impair the proper formation of myotubes; that is to say, there are more MHC positive mononucleated cells (myocytes) and fewer, smaller myotubes in cell lines overexpressing a newt gene. To quantify the data, a fusion index was calculated by dividing the number of nuclei within MHC positive multinucleate (and separately, mononucleate) cell bodies by the total number of nuclei (as visualized by DAPI staining). [Figure 22A](#) shows a significant drop in the fusion index in all cell lines overexpressing newt genes and an increase in the number of myocytes in C2C12s expressing newt *β -catenin* or *T β 4*. A Western blot was performed for MHC on protein from 96 hour MT samples ([Fig. 22B](#)) and quantification of MHC levels ([Fig. 22C](#)) shows a decrease in total levels of MHC in the C2C12s expressing newt genes.

Figure 22. Fusion index and Western blot for MHC expression in C2C12 myoblasts expressing newt β -catenin, wntless, thymosin- β 4, or dapper during differentiation.

(A) Fusion index (*i.e.*, percentage of nuclei in MHC positive myotubes) quantifies the decrease in myotube size (seen in [Fig. 21](#)) following expression of newt β -catenin, wntless, $T\beta 4$, and dapper. The percentage of MHC positive mononucleate cells also increases with expression of β -catenin and $T\beta 4$. (n=3; Student's T-Test, *p<0.05) **(B)** Western blot for MHC shows differences in the amount of MHC protein present in 96 hour MT C2C12 samples expressing the indicated newt gene. **(C)** Quantification of Western blot in **B**.

A**B****C**

DISCUSSION

4.1 – Major Contributions

Despite the fact that the incredible regenerative abilities of the newt have been known for almost 250 years (Gargioli and Slack, 2004; Odelberg, 2005; Whited and Tabin, 2009), we still know relatively little about the cellular and molecular processes underlying this phenomenon. The most important contribution of the work described in this thesis is the identification of approximately 410 genes ([Table 1](#)) with a potential role in the early events of newt forelimb regeneration. Of these genes, 270 revealed homology to known genes in gene and protein databases. Significantly, 140 of these 410 genes remain as “unknowns” and it is possible that some of these may represent novel newt genes important in the regeneration process.

The 410 genes were printed onto custom arrays and the expression profiles of these genes have been assessed during various stages of forelimb and tail regeneration using microarray analysis ([Fig. 7](#), [Tables 2](#) and [3](#)). The data from this analysis have helped to identify candidate genes, gene families, and signalling pathways that may be potential regulators of the dedifferentiation and regeneration processes.

The main roadblock preventing rapid progress in understanding more about regeneration in the newt is the lack of a fully sequenced and annotated genome, and thus, a lack of molecular tools with which to study this process. We believe our methodology represents one way to overcome this roadblock by, first, acquiring a large number of sequences specific to regeneration in the initial screens (using RDA) and then validating those sequences and analyzing their expression changes in various regenerating

organs/tissues (*e.g.*, via microarrays and qPCR). The latter experiments allow the detection of genes whose expression profiles are altered in regenerating tissues, identifying promising gene candidates for follow-up study. The validity of using this approach to identify genes important in regeneration is supported by recently published sequence and expression data using similar methods (*e.g.*, differential display analysis followed by microarrays; Bruckskotten *et al.*, 2012; Looso *et al.*, 2013; Mercer *et al.*, 2012).

In addition to the studies mentioned above, we have used RACE to identify the full length coding sequences (CDS) of five newt genes: β -catenin ([Fig. 8](#)), *wntless* ([Fig. 9](#)), *dapper* ([Fig. 10](#)), *T β 4* ([Fig. 11A](#)), and *T β 10/15* ([Fig. 11B](#)). We have also conducted a detailed analysis of the expression of these five genes throughout limb regeneration, during early tail regeneration, and during the differentiation of newt A2 myoblasts using a combination of qPCR ([Figs. 12](#) and [13](#)), IHC ([Figs. 15](#) and [16](#)), and ISH ([Fig. 17](#)). Furthermore we have initiated a functional analysis of four of these genes (*T β 10/15* excluded) by overexpressing them in mammalian C2C12 myoblasts and analyzing their effects on the growth ([Fig. 20](#)) and proper differentiation ([Figs. 21](#) and [22](#)) of these cells.

4.2 – RDA and Microarray

Our analysis of genes involved in early limb regeneration in *N. viridescens* has provided a list of 410 unique, differentially regulated, partial cDNAs ([Table 1](#)). These may represent fewer than 410 genes if some cDNA fragments belong to the same gene. Importantly, the RDA provided an unbiased approach to identify differentially expressed genes and the expression of many have been validated through both qPCR ([Fig. 6](#)) and microarray analysis ([Fig. 7](#), [Tables 2](#) and [3](#)). Our results are further validated by the

appearance in our analysis of several genes which have been shown previously, by other groups, to be involved in regeneration in newts. For example, newt *matrix metalloproteinase 3/10a* (*MMP3/10a*; [Fig.6A](#) and [Table 2](#)) was previously identified by Vinarsky *et al.* (2005) and their work suggested that MMPs are required for normal limb regeneration in *N. viridescens*. Our microarray data show that this gene is upregulated throughout all time points in the regenerating limb and there is no change in expression in the regenerating tail ([Table 2](#)). This may indicate either a higher basal level of expression in the tail, or perhaps *MMP3/10a* is not required for tail regeneration. The same group showed that an inhibitor of MMPs (*TIMP1*, *tissue inhibitor of MMP1*; [Fig. 6](#) and [Table 2](#)) is also present in the regenerating newt limb and may play a role in maintaining optimal levels of MMP activity during early limb regeneration (Stevenson *et al.*, 2006). Our microarray analysis also shows an upregulation of *TIMP1* throughout limb regeneration and a slight increase in expression at the early blastema stage (T7) of tail regeneration ([Table 2](#)). A newt *collagenase* was also identified in our screen (*Col*, [Table 2](#)) and it has been shown to be upregulated within 24 hours after limb amputation (Calve *et al.*, 2010; Vinarsky *et al.*, 2005). Our microarray data indicate no change in expression at 24 hours (R1), but it is upregulated by early dedifferentiation (R3) and throughout the remaining time points of limb regeneration ([Table 2](#)). Newt *collagenase* shows no change at any time during tail regeneration ([Table 2](#)). *Jumonji* family genes have been shown to play a role in chromatin regulation (*e.g.*, through histone demethylase activity) which may allow them to regulate gene expression and control development through various pathways (Takeuchi *et al.*, 2006). These epigenetic modifiers were also shown, through microarray analysis of dedifferentiating pigmented epithelial cells (PECs) from the newt iris during

lens regeneration, to be upregulated and thus, may play a role in dedifferentiation and/or transdifferentiation (Jopling *et al.*, 2011). We also identified an upregulated *jumonji* family member in our screen which was shown via microarray to be upregulated in the early limb blastema (R7) with no change during tail regeneration ([Table 2](#)). This may indicate a role for *jumonji* family genes in reprogramming the limb blastema cells for transdifferentiation.

Some genes appeared in our screen which have been shown to be involved in regeneration in other model organisms as well. Three such genes, *annexin1* (*ANXA1*), *annexin2* (*ANXA2*), and *S100A10* ([Table 2](#)), were also shown by King *et al.* (2009) to be upregulated in a protein screen comparing intact and three day regenerating limbs in larval *Xenopus*. Our microarray data show that, in the adult newt, these three genes are actually upregulated as early as the wound healing phase (R1) and, except for the early blastema phase (R7) in *ANXA1* and *S100A10*, remain upregulated throughout limb regeneration ([Table 2](#)). In the regenerating tail, only *S100A10* shows an upregulation at the early blastema stage (T7; [Table 2](#)). These genes are known to play a role in immune regulation and their upregulation during newt and *Xenopus* limb regeneration indicates a potential role for immune modulation during dedifferentiation. King *et al.* (2009) also discussed the upregulation of *keratin 17* (*KRT17*), which is strongly expressed in the wound epithelium, and its potential role in influencing cell growth and death via interactions with other non-structural proteins. *KRT17* was also identified as a highly upregulated gene in our R3 RDA library. Thirty-four of the original 1200 clones picked following the subtraction represented *KRT17*. It was validated by qPCR ([Fig. 6](#)) and

shown by microarray to be upregulated throughout limb regeneration and in the latter stages (T7 and T15) of tail regeneration ([Table 2](#)).

Our screen identified genes that have been linked to wound healing in mammalian systems. *Ob/Lep* (leptin), a hormone which normally regulates energy intake and expenditure, was upregulated during wound healing (R1) and in the early redifferentiation stage (R21) of the regenerating limb with no change observed in the regenerating tail ([Table 2](#)). According to several studies, leptin is also known to upregulate *vascular endothelial growth factor* (*VEGF*) expression (thus, promoting angiogenesis) and to promote wound healing in mice and rats subjected to ulcers, burns, or skin lesions (Seitz *et al.*, 2010; Tanigawa *et al.*, 2010; Wen *et al.*, 2012). These properties of leptin might explain our microarray data: it may be involved in promoting wound healing early in the regeneration process (R1) and promoting angiogenesis, via *VEGF* upregulation, later on (R21) in limb regeneration to help provide a blood supply to the growing limb bud. Another study showed that CD9, a cell surface protein, is important for wound re-epithelialization in mice and plays a role in regulating *MMP9* (Zhang *et al.*, 2012a). *CD9* expression levels were found to be upregulated and rising throughout limb regeneration, while unchanging throughout tail regeneration ([Table 2](#)).

In addition to identifying genes whose upregulation is necessary to promote the regeneration process, our screen also identified genes which may antagonize regeneration and whose downregulation is necessary for regeneration to progress. For example, we identified a downregulated gene, *apoptosis-inducing factor* (*AIFM1*, not shown), in our screen which, in the context of limb regeneration, may require downregulation to prevent the cell death of the newly dedifferentiated and proliferating cells. However, one group

has reported that *AIFM1* may be necessary for normal muscle regeneration in rats, although this only occurs when it exhibits ROS-generating NADH oxidase activity (Qiu *et al.*, 2008). Another gene, *FBXW11*, acts to prevent progression of the cell cycle during genotoxic stress until DNA repair has been completed. The microarray shows an upregulation of this gene only in the early dedifferentiation stage (R3) of limb regeneration ([Table 2](#)) which would be in line with its function, *i.e.*, preventing cells from cycling while they are being reprogrammed. However, this gene was originally identified in the R0 RDA library, marking it as a gene which is downregulated during regeneration. Downregulating *FBXW11* during limb regeneration (particularly during the blastema stage) could allow for the speedy production of new cells for the regenerate. Another gene with contradicting profiles based on qPCR and microarray analysis was *RAD51*, a protein involved in the repair of DNA double strand breaks. Quantitative PCR analysis showed that it is downregulated at the dedifferentiation stage (R3; [Fig. 6](#)) which would allow cells to divide quickly without DNA repair. However, the microarray data show upregulation of *RAD51* only at the dedifferentiation stage (R3; [Table 2](#)), suggesting a possible role in repairing DNA strand breaks in the initial blastema cells. It is difficult to reconcile the contradictory data obtained by the RDA/microarray/qPCR, but some of this may be explained by limitations in these techniques discussed below (see [Section 4.2.1](#)).

Heat shock proteins (HSPs) are a class of proteins whose expression is increased when cells are stressed. We identified three HSPs in our analysis (*HSP40*, *HSP70* and *HSP90*; [Fig. 6B](#) and [Table 2](#)) which are differentially regulated during regeneration. Upon inhibition of *HSP90* during mouse liver regeneration, Hackl *et al.* (2010) showed that *HSP70* was upregulated and regeneration ensued. Our results show the same pattern

occurring in newt limbs with *HSP90* being downregulated (according to the RDA) and *HSP70* upregulated throughout limb regeneration ([Fig. 6B](#) and [Table 2](#)). There is no change in *HSP70* during tail regeneration ([Table 2](#)). However, contradictory to reports of *HSP40* being upregulated during zebrafish fin regeneration (Tawk *et al.*, 2000), we find this gene to be downregulated during newt limb regeneration ([Table 2](#); or at least no change as per qPCR, [Fig. 6](#)). There is also no change in *HSP40* expression during tail regeneration ([Table 2](#)). This may indicate a different function for *HSP40* during newt regeneration compared to other model organisms.

4.2.1 – Limitations of RDA, Microarray, and qPCR Analysis

There are clear limitations associated with many of the techniques described in this thesis. For the limb regeneration data analysis, the first potential confounding factor could simply be the natural variation between animals. This variation can be caused by differences in age, health status, or size. We attempted to overcome this problem by including multiple animals (*i.e.*, at least ten) per time point; however, individual variation between animals may be enough to affect the isolated RNA pool. For example, if an animal being amputated for an R7 limb hasn't quite reached that stage (developmentally speaking), and if both of its amputated limbs make it into the pool of RNA transcripts, it may greatly affect the level of expression of a particular gene in that pool.

The second potential confounding factor in the limb regenerates could be the size of the amputated limb sample. The protocol involved the amputation of only the distal 1mm of the regenerating tip. However, when performing an amputation on such a small limb, it is likely that this measurement could potentially vary by as much as $\pm 0.5\text{mm}$. If this were the case, comparing an RNA sample from a 0.5mm stump to a 1.5mm stump

could indicate a marked change in the expression of a particular gene, by simply overwhelming the blastema tissue with excess stump tissue. It is likely that both of these confounding factors may have played a role in the variation we saw in our data, which led to difficulties in achieving statistical significance for many time points during limb and tail regeneration.

One potential hindrance to the analysis of our gene list is the inability to do gene pathway and network analysis. A reason for this may be the incomplete profile of gene expression provided by the RDA technique. Since only 1200 clones were picked and analyzed, it is probable that many genes were missed, especially ones with low expression levels. While the appearance of certain members of gene pathways can indicate a potential role for that pathway in regeneration, the remaining members must be identified in order to carry out a more complete analysis. It would have been preferable to analyze a larger number of clones (*e.g.*, >10,000); however, with limited resources available, we decided 1200 would be sufficient for initial analysis. Other groups have done similar work (Abdullayev *et al.*, 2013; Bruckskotten *et al.*, 2012; Looso *et al.*, 2013; Mercer *et al.*, 2012) using high throughput sequencing technologies to increase the number of transcripts analyzed, which provided them with a larger picture of the genetic interactions involved in regeneration than we were able to achieve.

Some of our data present contradictions in gene expression between the RDA, microarray, and/or qPCR. It is likely these contradictions stem from the limitations that are inherent to each technique. For example, through qPCR analysis, we validated only 67% of a representative sample of genes from the RDA. Microarray analysis only validated 56-65% of the RDA genes. Also, microarray analysis will only identify genes

with large changes in expression; in other words, changes in low expressing genes (*e.g.*, transcription factors) may be missed in the microarray. Quantitative PCR is more accurate in this case, but cannot be performed on as many genes at once. The integrity of the RNA is also crucial for hybridization to occur properly in microarrays and they can also be affected by the presence of multiple family members (*e.g.*, *Tβ* genes) in a sample. Quantitative PCR can be more specific, but will still be affected by heterogeneity in samples as described above, or slight variations in RNA integrity.

Some of our data also show differing profiles for genes between regenerating limb and tail tissue. This discrepancy could be due to the fact that, despite the shared ability to regenerate, the limb and tail are two completely different tissues. While both must regenerate muscle, bone, nervous tissue, etc., the tail contains spinal cord and the tissues are organized differently than in the limb. Furthermore, even if similar genes are involved in regeneration in the two tissues, the rates of regeneration may be different. For example, a gene which is upregulated at three days post-amputation in the limb may not become upregulated until seven days post-amputation in the tail. Overall, the purpose of the tail regeneration profile was to have another way of identifying similar expression patterns to the limb regeneration profile. This would indicate genes with a potentially shared role in regeneration in multiple tissues and provide further evidence for choosing potential candidates for follow-up study.

Finally, some of our data contradict what is seen in the literature. Sometimes this can be attributed to the use of different model organisms (*e.g.*, fish, frogs, or axolotls instead of newts). Alternatively, it may be due to the fact that tissue sampling was done

differently; as mentioned above, amputating a bit more (or less) stump tissue can affect the regeneration profile.

4.3 – Analysis of Genes of the Wnt Signalling Pathway and Thymosin- β During Limb/Tail Regeneration and A2 Myoblast Differentiation

Three genes of the wnt signalling pathway (*β -catenin*, *wntless*, and *dapper*) were identified in the RDA. Since they are all part of the same pathway, and since this pathway has been implicated in stem cell renewal and amplification, we decided to begin our detailed analysis with these genes. Additionally, *thymosin- β* family members were identified by the microarray as the most highly upregulated genes throughout limb and tail regeneration ([Table 2](#)). Based on the expression data and on reports indicating *T β 4* is a novel regulator of wnt signalling (Goldstein *et al.*, 2012; Ryu *et al.*, 2012), we decided to include *T β 4* and *T β 10/15* in this initial analysis.

4.3.1 – Comparison of Protein Sequences

Using RACE to obtain the full length CDS for each gene allowed us to compare the protein sequences of the newt genes to those of other vertebrates, including the zebrafish (*Danio rerio*), frog (*Xenopus sp.*), chicken (*Gallus gallus*), mouse (*Mus musculus*), and human (*Homo sapiens*) sequences. This first step was important to determine if there were any major differences in the protein coding region which may be shared in species that exhibit more extensive regenerative abilities (*e.g.*, fish, frog, and newt) versus those with less regenerative prowess (*e.g.*, chicken, mouse, and human). The reasoning behind this was if the proteins in regenerating species share common

differences from those in non-regenerators, it may allude to a shared, modified function of the protein. Alternatively, if the newt protein alone diverges from the other species, it may indicate a novel regulation or function of the newt protein. Another potential explanation for any divergence in protein sequence could simply be that the divergent sequence does not change the function of a critical part of the protein and, therefore, is not selected for during evolution.

Four of the five genes (*β-catenin*, *wntless*, *Tβ4*, and *Tβ10/15*) had protein sequences which were very highly conserved among the vertebrate species tested ([Figs. 8, 9, and 11](#)). Only *dapper* showed some divergence at the protein level, particularly in the newt and towards the N-terminus ([Fig. 10](#)). This may indicate that a novel function or regulation is available to the newt dapper protein which may play a role in the dedifferentiation and regeneration process. Alternatively, it may indicate that the crucial functional domains of this protein are at the C-terminus, allowing variation at the N-terminus without loss of function.

4.3.2 – Analysis of Genes of the Wnt Signalling Pathway

We performed qPCR on the genes of the wnt signalling pathway during limb and tail regeneration to determine their expression profiles and the potential for these genes to be involved in regenerative processes. Time points for limb regeneration were chosen to reflect key aspects of the regeneration process: wound healing (R1), peak dedifferentiation (R3, R5), early blastema (R7), mid-blastema (R15), and late blastema/early redifferentiation (R21; see Iten and Bryant, 1973 for staging). For tail regeneration, only the three day peak dedifferentiation time point (T3) was tested as it proved extremely difficult to obtain sufficient amounts of RNA from tail tissue. Both

limb and tail samples were compared to their intact tissue control (R0 and T0, respectively).

The qPCR data indicated that *dapper* and β -*catenin* may have antagonistic roles during limb regeneration ([Fig. 12A](#)). As *dapper* slowly increases throughout regeneration, β -*catenin* trends downward following a peak at R3. This is in line with the traditional role of *dapper* as an antagonist of β -*catenin* (Cheyette *et al.*, 2002). Unfortunately, the R3 qPCR data did not validate the RDA results for *dapper*, which showed that it was downregulated. This may be due to the limitations associated with these techniques, as previously described (see [Section 4.2.1](#)). During tail regeneration ([Fig. 12B](#)), *dapper* trended downwards while β -*catenin* was upregulated, as predicted by the RDA. In the context of both limb and tail regeneration, the inverse relationship between *dapper* and β -*catenin* makes sense in that β -*catenin* plays a role in initiating cell cycling, stem cell maintenance, and even regulation of extra-cellular matrix remodelling proteins (*i.e.*, MMPs; Brabletz *et al.*, 1999; Crawford *et al.*, 1999; He *et al.*, 1998; Ingraham *et al.*, 2011; Liu *et al.*, 2011; Shtutman *et al.*, 1999; Tetsu and McCormick, 1999; Wu *et al.*, 2007; Zhang *et al.*, 2012b). Therefore, it would need to be upregulated during the early phases of limb regeneration where there is much reorganization of the stump tissue and dedifferentiating cells need to begin proliferating in order to form the blastema (Yokoyama, 2008). In support of this, studies have pointed towards a role for β -*catenin* signalling in the initiation of limb regeneration and blastema formation (Kawakami *et al.*, 2006; Lin and Slack, 2008; Yokoyama *et al.*, 2007; Zhang *et al.*, 2012b).

Towards the latter stages of regeneration, it would make sense for β -catenin signalling to reduce and for *dapper* expression to increase, as is seen in [Fig. 12A](#). At this point, cells are leaving the blastema and exiting the cell cycle to start redifferentiating into mature tissue (Iten and Bryant, 1973). Upregulation of *dapper*, working as an antagonist of β -catenin, would aid in reducing the expression of downstream targets of β -catenin (i.e., cyclins, MMPs, etc.).

The above qPCR data for β -catenin are strengthened by the IHC analysis. We stained limb sections with an anti- β -catenin antibody to determine where the protein was localized in the regenerating limb. [Figure 15](#) clearly shows high levels of β -catenin protein in the R3 limb at the plane of amputation as well as more proximally ([Fig. 15D](#)). As regeneration proceeds, the β -catenin protein becomes less prominent and more localized to the blastema ([Fig. 15E-G](#)), which is in line with the expression levels indicated by the qPCR ([Fig. 12A](#)).

Similar to *dapper*, qPCR data for *wntless* indicated a gradual increase in transcript levels throughout limb regeneration ([Fig. 12A](#)). *Wntless* is a membrane protein with several transmembrane domains, similar to G-protein coupled receptors (GPCR), although there is no evidence that *wntless* signals as a GPCR (Bänziger *et al.*, 2006; Herr and Basler, 2012; Zhu *et al.*, 2012). It is involved in the transport of wnts and in their secretion from cells to promote wnt signalling, and studies suggest it binds to Wnt1, Wnt3a, and Wnt5a, indicating a role in both canonical and non-canonical wnt signalling (Bänziger *et al.*, 2006; Herr and Basler, 2012; Zhu *et al.*, 2012). *Wntless* has also been shown to be involved in distal limb patterning (Zhu *et al.*, 2012) and is required for proper wnt secretion and, ultimately, proper regeneration in planarians (Adell *et al.*,

2009). Thus, the expression profile makes sense in the context of limb regeneration: *wntless* would need to be upregulated as regeneration proceeds, particularly towards the redifferentiation stage, to aid in distal limb patterning.

We also conducted a qPCR analysis on A2 myoblasts during differentiation, comparing sub-confluent myoblast (SCMB) controls to confluent myoblasts (CMB) and myotube (MT) cultures after four days in low-serum, differentiation media. The A2 cells were generated in order to replace the ageing A1 cell line which had previously been shown to re-enter the cell cycle following serum stimulation. Thus, our intent was to use A2 cells in dedifferentiation assays. A2 muscle cells differentiate normally and form multinucleated myotubes similar to A1s; however, we determined there was no cell cycle re-entry within the first ten passages. In subsequent passages, the A2 myotubes were found to re-enter the cell cycle following serum stimulation, but this was lost again at a higher passage number (*e.g.*, more than 25 passages). Thus, we believe the assumption that cell cycle re-entry is a normal property of newt myotubes is erroneous, and instead, is a trait which may have been acquired in culture. This may suggest that newt A1 cells acquired mutations in culture that allow them to re-enter the cell cycle, and the fact that this was obtained in an A2 clone as well suggests that newt cells are “primed” for cell cycle re-entry with fewer “checkpoints” than mammalian cells.

Even though we couldn’t assess cell cycle re-entry in A2 cells, we wanted to test the level of expression of the genes of the wnt signalling pathway throughout myogenesis. The reasoning behind this is that genes which would play a role in promoting differentiation (*i.e.*, myogenesis) would most likely be downregulated to allow for dedifferentiation and regeneration (and *vice versa*). The qPCR data indicated that β -

catenin appeared to be upregulated as A2 myoblasts differentiated into myotubes and *dapper* appeared to be downregulated (Fig. 12C). Although β -*catenin* plays a role in proliferation and myoblasts must exit the cell cycle to differentiate into myotubes, the protein also plays a functional role in myoblast fusion in association with M-cadherin (Wang *et al.*, 2013; Wróbel *et al.*, 2007). In this respect, an increase in β -*catenin* transcript may be warranted in order to produce more protein for a new structural role during differentiation. The data from the qPCR indicated no change in the levels of *wntless* expression throughout A2 myoblast differentiation.

4.3.3 – *Thymosin- β* Gene Analysis

Quantitative PCR experiments on limb/tail regeneration and A2 differentiation were also conducted for *T β 4* and *T β 10/15*. The qPCR data for limb regeneration indicated a slow increase in *T β 10/15* levels, but only becoming significantly upregulated by R15 compared to R1 (Fig. 13A). On the other hand, *T β 4* transcript levels were shown to rise rapidly in the regenerating limb (Fig. 13A). Interestingly, the three day profiles in the tail (T3) showed no change in *T β 4* while *T β 10/15* was significantly upregulated (Fig. 13B). This may indicate a tissue specific role for these two members of the *thymosin- β* family, with *T β 10/15* playing a more prominent role in the tail and *T β 4* playing a greater role in the limb during regeneration. The data, at least for *T β 4*, are in line with other research indicating a role for this gene in wound healing, prevention of scar formation, and attracting cells to the site of regeneration (Ehrlich and Hazard, 2010; Goldstein *et al.*, 2012). In the newt limb, which heals quickly, exhibits no scarring, and must attract dedifferentiating cells to the blastema for regeneration (Yokoyama, 2008), it makes sense that *T β 4* would be upregulated early and stay up throughout regeneration.

As with *β-catenin*, the qPCR data for *Tβ4* and *Tβ10/15* are strengthened by the IHC, and in this case, ISH analysis. Limb sections were stained with an anti-Thymosin-β-4 antibody to determine where the protein was localized in the regenerating limb. [Figure 16](#) clearly shows increasing levels of Tβ4 protein from R3 to R21 ([Fig. 16C-F](#)). As regeneration proceeds, the Tβ4 protein becomes more prominent and spreads from the plane of amputation to more proximal areas of the regenerating limb ([Fig. 16C-F](#)), which is in line with the expression levels indicated by the qPCR ([Fig. 13A](#)).

Because the specificity of the antibody used for Tβ4 localization wasn't confirmed via Western blot, and since Tβ4 and Tβ10/15 have high homology at the protein level, we performed ISH to attempt to separate the staining pattern of the transcripts for *Tβ4* and *Tβ10/15*. This analysis would indicate whether the antibody was specific to Tβ4 or Tβ10/15 in the IHC studies. As expected, staining for *Tβ4* transcript was present and specific to the blastema in the R7 limb ([Fig. 17A](#)), while *Tβ10/15* transcript was not present in the R7 limb blastema ([Fig. 17C](#)). These data agree with the qPCR profiles of both genes and the IHC analysis of Tβ4. As proof that the *Tβ10/15* probe had indeed worked, it was shown to stain the muscle tissue of an R3 limb, proximal to the plane of amputation ([Fig. 17D](#)).

The expression profiles of the *thymosin-β* genes were also analyzed in the differentiating A2 myoblasts. The qPCR data indicated a downward trend during differentiation for both *Tβ10/15* and *Tβ4*, with the latter significantly downregulated in myotube cultures ([Fig. 13C](#)). These data fit with what is expected based on the roles *Tβ4* is known to play in cell migration and proliferation (Ehrlich and Hazard, 2010; Goldstein *et al.*, 2012; Philp and Kleinman, 2010).

4.3.4 – Limitations with qPCR and IHC/ISH Analysis

The main difficulty in analyzing the results from the qPCR analysis has been accounting for the large variation between biological replicates that was seen in the data sets (see [Section 4.2.1](#)). Many of the data points in the qPCR histograms do not indicate statistical significance because the error in the samples is quite large. Despite this, we believe that the overall trends for the data are correct as they have been outlined and discussed because the majority of individual experiments showed the same trends.

Similarly, we believe that some of the variation we saw in the qPCR data for A2 myoblast differentiation could come from differences in the precise level of myogenesis occurring on different plates. That is to say, a dish of confluent myoblasts (CMB) or myotubes (MT) is not a homogeneous cell population. For example, a CMB plate which has an area (or areas) that became confluent sooner, may start to differentiate prematurely in those areas (*i.e.*, without the addition of low-serum media). Contrasting this with a plate that was seeded more evenly and, as such, reached confluency more evenly, this might indicate differences in the level of a particular gene's expression within the same time point. Similarly, not all of the myoblasts in a plate fuse to form myotubes in MT cultures, even at four days of differentiation. Differences in the precise number of myotubes versus myoblasts could create differences in the RNA pools of separate plates.

In terms of the IHC and ISH studies, there were also limitations. Wntless and dapper antibodies were tested on newt tissues using Western blot analysis, but they proved to be non-specific, with multiple bands appearing on the blots. There were also limitations provided by the quality of the sections obtained for histology. Sectioning such a small limb and obtaining an ideal longitudinal section (*i.e.*, a section through the

blastema and bone with all three axes lined up) is a difficult task, at best. When trying to localize the proteins and transcripts in this snapshot of a three-dimensional environment, it is apparent that not all of the sections contain all of the required information. Thus, multiple sections were observed before making any of the conclusions described here.

4.3.5 – Summary of Expression Analyses

The data we have provided above indicate a role for genes of the wnt signalling pathway and *thymosin- β* (specifically *T β 4*) during dedifferentiation and regeneration in *N. viridescens*. Although we have yet to obtain functional evidence in the newt, the expression and localization of the genes we have identified are clearly correlated with specific stages of limb regeneration ([Figs. 12A](#), [13A](#), [15](#), [16](#), and [17](#)). Additionally, evidence for a functional role of these genes and signalling pathways during wound healing and regeneration has been shown in other species (Ehrlich and Hazard, 2010; Goldstein *et al.*, 2012; Kawakami *et al.*, 2006; Lin and Slack, 2008; Yokoyama *et al.*, 2007; Zhang *et al.*, 2012b). Taken together, we believe this makes a case for wnt signalling and *T β 4* playing a role in dedifferentiation and regeneration in the newt.

4.4 – Analysis of Newt β -catenin, Wntless, Dapper, and Thymosin- β 4 Expression During C2C12 Myoblast Differentiation

The original goal was to transfect the newt genes we had identified into newt A2 myoblasts to conduct a functional analysis of these genes during the dedifferentiation process. Unfortunately, these cells are notoriously hard to transfect (Kumar *et al.*, 2000) and, despite our best efforts with multiple transfection tools (various lipophilic reagents and electroporation) and viral vectors (lentivirus and adeno-associated virus), we were

never able to achieve adequate transfection of these cells. Furthermore, the A2 cells cycle slowly and do not respond well to selection under G418. After many months of selection and attempting to expand the cells, the cultures simply became senescent.

In order to obtain some functional data, we decided to transfect the newt genes into mouse C2C12 myoblasts. While these cells do not exhibit the partial dedifferentiation response shown by A1 myotubes (*i.e.*, cell cycle re-entry upon high-serum stimulation), they do follow a conserved differentiation program (Kislinger *et al.*, 2005; Straube and Tanaka, 2006; Tannu *et al.*, 2004). With this in mind, we decided to test whether any of the newt genes were able to disrupt the differentiation program in C2C12s.

4.4.1 – Expression Analysis of Mouse β -catenin, Wntless, Dapper, and Thymosin- β 4 in Untransfected C2C12 Myoblasts

Prior to working with transfected C2C12s, we wanted to first determine the normal expression pattern of β -catenin, wntless, dapper, and *T β 4* during C2C12 myoblast differentiation [including changes from sub-confluent myoblasts (SCMB), to confluent myoblasts (CMB), and through four days of myotube differentiation (24, 48, and 96 hour MT); [Fig. 18](#)]. All three genes of the wnt signalling pathway showed either significant upregulation or indicated upregulation throughout the differentiation process, with β -catenin peaking at the 24 hour MT stage ([Fig. 18A](#)), wntless peaking at the CMB stage and levelling off in MTs ([Fig. 18B](#)), and dapper indicating a peak at the 24 hour MT stage ([Fig. 18C](#)). Comparing these results to [Fig. 12C](#) (*i.e.*, wnt signalling pathway gene expression during A2 myoblast differentiation), one can see some differences in the trends between newt A2 and mouse C2C12 myoblast differentiation. In C2C12 cells, β -

catenin is significantly upregulated by the MT stage, while in A2 cells, it only appears to be upregulated, although not significantly, at the same stage. This may reflect the multiple roles that β -*catenin* plays during muscle differentiation, including both cell cycling and a structural role in myoblast fusion (Goichberg *et al.*, 2001; Shtutman *et al.*, 1999; Tetsu and McCormick, 1999; Wang *et al.*, 2013; Wróbel *et al.*, 2007). *Wntless* expression appears unchanged in A2 cells and, while it is fairly stable in MTs during C2C12 differentiation, it is significantly upregulated throughout. This may reflect the fact that *wntless* is associated with differentiation in other systems (Zhu *et al.*, 2012). The differences observed in *wntless* expression between A2 and C2C12 cells may be explained by differences in the degree of differentiation and cell fusion, with one having more contaminating myoblasts than the other. One major discrepancy between A2 and C2C12 cells is between *dapper* levels, which indicate a decrease throughout A2 differentiation (significant in the CMB stage), but an increase throughout C2C12 differentiation. If *dapper* is only involved in regulating β -*catenin* as a transcription factor, the C2C12 profile would make sense because, as cells exit the cell cycle, the increased levels of *dapper* would prevent the action of β -*catenin* on TCF/LEF target genes would be reduced. However, if *dapper* has additional functions in newt cells, and is also involved in regulating β -*catenin* structural roles in myoblast differentiation, then a reduction in *dapper* would be necessary for proper differentiation to occur.

It is also possible that these discrepancies could be attributed to the variance in the differentiating cultures (seen mostly in the A2 CMB and MT cultures; see [Section 4.3.4](#)). Although, it may be possible that the sequence differences exhibited by *dapper* (shown at

the protein level in [Fig. 10](#)) could play a role in the altered expression exhibited between newt and mouse muscle cells.

Examining the expression of *Tβ4* during C2C12 myoblast differentiation, [Fig. 18A](#) indicated a small rise (~1.6x) in *Tβ4* expression at the CMB stage followed by a drop to control levels throughout the MT stages. There is, again, a slight discrepancy between newt and mouse cells, with [Fig. 13C](#) showing a two-fold downregulation of *Tβ4* in A2 MT cultures. It is difficult to predict whether the modest (but significant) differences seen in *Tβ4* levels between A2 and C2C12 cells would be physiologically relevant. As above, this discrepancy may be an issue with the heterogeneity that can be found in differentiating C2C12 and A2 cultures.

We believe the data for the C2C12 differentiating cultures are valid as the threshold cycle (C_T) values from the qPCR were both accurate and precise, thus reducing the error bars significantly (compared to C_T values for A2 cultures which were accurate, but less precise). In other words, the C2C12 data had only small variations both within (*i.e.*, accurate) and between experiments (*i.e.*, precise), while the A2 data had small variations within experiments, but larger variation between experiments. This makes comparing the two difficult as the data for the A2 differentiating cultures, while still valid, have a much larger error.

4.4.2 – Expression Analysis of Newt *β-catenin*, *Wntless*, *Dapper*, and *Thymosin-β 4* in Transfected C2C12 Myoblasts

Following the transfection of C2C12 myoblasts with the newt genes and selection to create stable cell lines, we performed qPCR on populations of sub-confluent myoblasts (SCMB) to determine if the newt genes were indeed being overexpressed in the mouse

cells ([Fig. 19](#)). To do this, primers were designed to detect both the endogenous mouse and exogenous newt versions of the gene of interest (referred to as ‘total’ gene expression). We also used these primers to detect the level of expression of the mouse versions of the other genes in the cell lines (*e.g.*, in the newt *dapper* transfected cell line, the primers for *dapper* would detect both mouse and newt transcripts, while the remaining primer sets would detect the levels of the mouse transcript for *β-catenin*, *wntless*, and *Tβ4* in this specific cell line).

Unfortunately, only newt *dapper* and *Tβ4* transfected C2C12 myoblasts indicated total expression levels for these genes to be upregulated ([Fig. 19B and C](#), respectively). The levels of total *β-catenin* were unchanged in newt *β-catenin* transfected C2C12s ([Fig. 19A](#)) and total *wntless* transcript actually appeared to be downregulated in newt *wntless* transfected mouse myoblasts ([Fig. 19D](#)). This might indicate a potential self-regulatory mechanism induced by the overexpression of the newt version of these two genes in the mouse cells which keeps the transcript levels in check. Interestingly, despite the lack of transcript overexpression, both the newt *β-catenin* and *wntless* transfected C2C12 cell lines exhibit altered proliferation and differentiation phenotypes (see [Section 4.4.3](#) and [Section 4.4.4](#); [Fig. 20A and B](#); [Fig. 21B and C](#); [Fig. 22](#)). These data, taken together, indicate that newt *β-catenin* and *wntless* are being transcribed and having an effect on the C2C12 myoblasts. It would be interesting to examine protein levels in these cell lines, as the stability of the proteins may affect the transcript levels during the differentiation process.

4.4.3 – Proliferation Assay on Transfected C2C12 Myoblasts

To test if any of the transfected newt genes could alter the growth of the C2C12 myoblasts, we performed a proliferation assay ([Fig. 20A](#)). The assay indicated that newt *β-catenin* transfected C2C12s proliferated faster than pCI (empty vector) control cells, while the newt *wntless* transfected cells grew slower. There was no significant difference in the newt *Tβ4* and *dapper* overexpressing lines compared to the control. The data for *β-catenin* are in line with the literature as some downstream targets of *β-catenin* are cell cycle genes (He *et al.*, 1998; Shtutman *et al.*, 1999; Tetsu and McCormick, 1999), and qPCR data for mouse *cyclinD1* indicated that it was upregulated in the newt *β-catenin* transfected C2C12s ([Fig. 20B](#)). To our knowledge, the specific role of *wntless* in cell cycle progression has not been studied, but its role in distal limb patterning and differentiation in other cell types may suggest a role in cell cycle exit. Additionally, we measured cell death during the proliferation assay to ensure the reduction in *wntless* transfected cells was due to a change in proliferation and not cell death. As is seen in [Fig. 20C](#), none of the transfected newt genes caused significant death in the C2C12 myoblasts at any time point over three days.

4.4.4 – Differentiation Assay on Transfected C2C12 Myoblasts

As a first step to elucidating a functional role for newt *β-catenin*, *wntless*, *dapper*, or *Tβ4* in dedifferentiation or regeneration, we decided to determine if the expression of any of the newt genes had an effect on the differentiation of mouse myoblasts. [Figure 21](#) shows that expression of each individual newt gene inhibited the proper differentiation of C2C12 myoblasts into myotubes. In each case, the myotubes that were present in cultures transfected with a newt gene were much smaller (in terms of number of nuclei

per myotube). In the case of C2C12s transfected with newt *β-catenin* and *Tβ4*, the number of myocytes (mononucleated cells expressing MHC) was increased, which may indicate either premature differentiation of myoblasts or failure of myoblasts to fuse and form proper myotubes. These data were quantified by calculating a fusion index ([Fig. 22A](#)). For further evidence indicating an effect on differentiation, a Western blot was performed to determine the levels of MHC in 96 hour MT cultures of the various C2C12 cell lines ([Fig. 22B](#) and quantified in [C](#)). Again, the overall drop in MHC levels in each cell line compared to the control indicated an impairment of differentiation in C2C12 myoblasts.

These data may suggest a role for newt *β-catenin*, *wntless*, *dapper*, and *Tβ4* in dedifferentiation and regeneration by showing that their expression in mouse myoblasts can inhibit proper differentiation into myotubes. This may be indicative of their potential roles in inducing the dedifferentiation of cells or helping to maintain cells in a dedifferentiated and proliferative state during blastema formation in newts.

4.4.5 – Summary of Overexpression Analyses

The analysis of the effect of expressing newt *β-catenin*, *wntless*, *dapper*, and *Tβ4* in C2C12 myoblasts has provided us with further evidence to implicate these genes in the dedifferentiation and regeneration process in newts. The effects of *β-catenin* in promoting proliferation and inhibiting proper differentiation fit perfectly in line with a role in initiating or promoting the dedifferentiation process. Similar roles for wnt signalling genes have been identified in other species (Kawakami *et al.*, 2006; Lin and Slack, 2008; Yokoyama *et al.*, 2007; Zhang *et al.*, 2012b). Similarly, the inhibition of proper differentiation by *Tβ4* is in line with its role in wound healing and regeneration

described in other species (Ehrlich and Hazard, 2010; Goldstein *et al.*, 2012). *Dapper*, which shows the least severe phenotype when overexpressed in C2C12 myoblasts, can clearly affect the differentiation process, although its role is more likely to counteract the expression of β -catenin in the wnt signalling pathway (Cheyette *et al.*, 2002). Finally, *wntless* may regulate cell cycle exit and, in doing so prematurely, may impact on the proper differentiation of myotubes.

4.5 – Overall Conclusions

Our work has contributed to the discovery of approximately 410 novel newt cDNAs and we have provided an analysis of the expression of these cDNAs during regeneration in multiple tissues. We have also provided an analysis of members of the newt wnt signalling pathway (β -catenin, *wntless*, and *dapper*) and the newt *thymosin- β* gene family (*T β 4* and *T β 10/15*) during newt limb/tail regeneration and A2 myoblast differentiation. Furthermore, we have initiated a functional analysis for four of these newt genes (*T β 10/15* excluded) following their transfection into mouse C2C12 myoblasts.

Our data has also provided a framework from which much more work can be initiated. Our gene list contains many interesting candidates which require follow-up to elucidate their precise roles in the dedifferentiation and regeneration processes. Our detailed analysis of the five newt genes has also provided a first insight into the potential roles of these specific genes during regeneration in *N. viridescens*, specifically, and laid the foundation for further functional analyses to be carried out.

This thesis is an important contribution to the understanding of the early genetic events of newt forelimb regeneration. Increasing our understanding of this unique

process could further human regenerative medicine by aiding in the discovery of new therapeutic targets for human health issues ranging from degenerative diseases, to spinal cord injuries, myocardial infarction, developmental pathologies, ageing, cancer, and even limb amputations.

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APPENDICES

Appendix I – Thermocycling Parameters

Bubble PCR

Primary PCR:

95°C/10 min
30 cycles:
 95°C/15 sec
 57°C/1 min
 72°C/30 sec
72°C/10 min
14°C/hold

Secondary PCR:

95°C/10 min
35 cycles:
 95°C/15 sec
 57°C/1 min
 72°C/30 sec
72°C/10 min
14°C/hold

Colony PCR

95°C/10 min
40 cycles:
 95°C/15 sec
 60°C/1 min
 72°C/30 sec
72°C/10 min
14°C/hold

Quantitative PCR

95°C/10 min

40 cycles:

95°C/15 sec

~57-62°C*/30 sec (*annealing temperature depends on primer pair T_m)

72°C/30 sec

72°C/10 min

14°C/hold

RACE PCR

1 cycle:

94°C/5 min

52°C/5 min

72°C/40 min

11 cycles:

94°C/40 sec

*57°C/1 min (*temperature decreases 0.5°C/cycle)

72°C/3 min

19 cycles:

94°C/40 sec

52°C/1 min

72°C/3 min

1 cycle:

94°C/40 sec

52°C/1 min

72°C/15 min

14°C/hold

Appendix II – Primer Sequences

*Y = C/T *R = A/G *N = A/C/T/G

Bubble PCR Primers

Bubble Bottom – 5' {CTC TCC CTT CTG CGG CCG CAG TTC GTC AAC ATA GCA TTT CTG TCC TCT CCT TC} 3'

Bubble Inner Primer (SP) – 5' {CGC TGT CCT CTC CTT C} 3'

Bubble Outer Primer (PP) – 5' {GCG GCC GCA GTT CGT CAA CAT AGC ATT TCT} 3'

Bubble Top – 5' {GAA GGA GAG GAC GCT GTC TGT CGA AGG TAA GGA ACG GAC GAG AGA AGG GAG AG} 3'

General Primers (Sequencing, Colony PCR)

M13 Forward – 5' {CGC CAG GGT TTT CCC AGT CAC GAC} 3'

M13 Reverse – 5' {TCA CAC AGG AAA CAG CTA TGA C} 3'

SP6 – 5' {GAT TTA GGT GAC ACT ATA G} 3'

T3 – 5' {ATT AAC CCT CAC TAA AGG GA} 3'

T7 Promoter – 5' {TAA TAC GAC TCA CTA TAG GG} 3'

ISH RNA Probe Primers

Tβ4 (F) – 5' {TCA CAG GGA TCA AGG CTT CT} 3'

Tβ4 (R) – 5' {CCT TTG ACA CTA GGC TCT GC} 3'

Tβ10/15 (F) – 5' {GCA GAC CTC TCT TTG GTG GA} 3'

Tβ10/15 (R) – 5' {GTC CGT CAC TAA CCA AAC AC} 3'

Quantitative PCR Primers

G003 (F) – 5' {TGC TGG GAC ACT CAC GCT CA} 3'

G003 (R) – 5' {ACC CAG ACA TTG TGA CAG TGA AGG A} 3'

G004 (F) – 5' {CTC CTC CCG CGA GCA GGT TC} 3'

G004 (R) – 5' {CAT AGC CCA ATG CAC GCC GC} 3'

G221 (F) – 5' {CGG GGA TGG CAG CGG AGT TG} 3'

G221 (R) – 5' {AGG GCA GGG TAG GAT GGA GCG} 3'

G224 (F) – 5' {CCA ACC CCG CAT GCT CAC ACT} 3'

G224 (R) – 5' {TAG CCC CAC TTC CCA GCC GT} 3'

Mmβcatenin (F) – 5' {GAG CCG TCA GTG CAG GAG} 3'

Mmβcatenin (R) – 5' {CAG CTT GAG TAG CCA TTG TCC} 3'

MmDapper (F) – 5' {CTG AGT GAT GGA GCT TCG GG} 3'

MmDapper (R) – 5' {GCC GCC TTT ACA TTC CAA CC} 3'

MmCyclinD1 (F) – 5' {CGG ATG AGA ACA AGC AGA CC}3'
MmCyclinD1 (R) – 5' {GAG GGT GGG TTG GAA ATG A}3'
MmGAPDH (F) – 5' {CGT CCC GTA GAC AAA ATG GT}3'
MmGAPDH (R) – 5' {CTC CTG GAA GAT GGT GAT GG}3'
MmHPRT (F) – 5' {GCAAACCTTTGCTTTCCCTGGTT}3'
MmHPRT (R) – 5' {CAAGGGCATATCCAACAACA}3'
MmTβ4 (F) – 5' {ACT CTC CTC GTT CGC GCA GC}3'
MmTβ4 (R) – 5' {GGC GGC GCT CGC CTC ATT A}3'
MmWntless (F) – 5' {TCC CTG GGT TAC CGT GAT GA}3'
MmWntless (R) – 5' {TAA CGA CCT TCG TGC TCT GG}3'
Mm/Nvβcatenin (F) – 5' {CGC ACC ATG CAG AAT ACA AAT GAT G}3'
Mm/Nvβcatenin (R) – 5' {GCT CTC TTG RTT GCC ATA AGC TAA}3'
Mm/NvDapper (F) – 5' {AGG AGA AGA GAT GCY GGT TTG TTG A}3'
Mm/NvDapper (R) – 5' {TCC AAG GGG CTG CMG AAG C}3'
Mm/NvTβ4 (F) – 5' {ATG TCT GAC AAA CCC GAT ATG G}3'
Mm/NvTβ4 (R) – 5' {GAA GGC AAT GCT YGT GGA ATG TAC}3'
Mm/NvWntless (F) – 5' {ATT GGA TTT RAC TGG ACC TGG ATG}3'
Mm/NvWntless (R) – 5' {GGT CCA ACT TGC TTC CAG TA}3'
Nv28SrRNA (F) – 5' {CGG ATG ACT GCC TCC GGT GC}3'
Nv28SrRNA (R) – 5' {ACA CAG CCC TTT GAC AGC GTG G}3'
NvABP25 (F) – 5' {ACC TCG CGC GGA AAG AAG GC}3'
NvABP25 (R) – 5' {AAC GCC TGC ACC AAC CGG AG}3'
NvAIFM1 (F) – 5' {GTG GTG CCT TTG CCG ACG TG}3'
NvAIFM1 (R) – 5' {CGC TCC AAC ATC TGG GTG GCA}3'
NvAKR1CL1 (F) – 5' {AGC CTC GCC AAC CTC CAC CT}3'
NvAKR1CL1 (R) – 5' {TCT GCA GAA GTG TGC GGC GG}3'
NvANXA1 (F) – 5' {TGA GCA TCA AAC TGC GCT GGG G}3'
NvANXA1 (R) – 5' {ATG CGG CTG CCC TCG ACA AG}3'
NvANXA2 (F) – 5' {GGC CTT TTA CCC TTT GCC AAC GC}3'
NvANXA2 (R) – 5' {GCC AGC GAG CTG AAG GGT TCC}3'
Nvβcatenin (F) – 5' {TCA GGG AAG GTG CTG TGG TGG T}3'
Nvβcatenin (R) – 5' {CGG GAA GAA GTA GCG GCA GG}3'
NvCBX3 (F) – 5' {GAT GGC AGA GTT AAA GCT TTC TGC G}3'
NvCBX3 (R) – 5' {AAC ATT CCC AGG CAG CCC GT}3'
NvCCNDBP1 (F) – 5' {CCA GCT CAT CAA CGC TGG GGC}3'
NvCCNDBP1 (R) – 5' {TGG GGG CTC GGG GTA ATC GG}3'
NvCOP9 (F) – 5' {TGC TTT TGT CCG GTT TCC AAC GG}3'
NvCOP9 (R) – 5' {CGC ACC TTC CTA CTC TGC CCG}3'
NvCytokeratinTypeII (F) – 5' {GGG AGT CAG TAA GCT CTG GTT GAC A}3'
NvCytokeratinTypeII (R) – 5' {TTG GTG GAG GAC CAG GCA ACA}3'
NvDapper (F) – 5' {CTG TGG CAG TGG CGG TTT GC}3'
NvDapper (R) – 5' {ACT GTG GTG GAC ACC AGC GA}3'
NvElafin (F) – 5' {CCG CTG AGA AAC CCG GGC AG}3'
NvElafin (R) – 5' {GCT TTG CGC TCA CAC AAG GCG}3'
NvEno1 (F) – 5' {AGA TTC CGG TGG AGG CCC CG}3'
NvEno1 (R) – 5' {GGG CCC CAC TGA ATT TGC TGC}3'

NvEP37-L2 (F) – 5' {TGC TTT CAT TGC ATT GCA GCT CTG A}3'
NvEP37-L2 (R) – 5' {TGG AAG GAA GGC ACA GAA CCA ACA}3'
NvEpidermalDifferentiationSpecificProtein (F) – 5' {AAG GCA CCA CCC CGC ACA AA}3'
NvEpidermalDifferentiationSpecificProtein (R) – 5' {GTC AGT TTT ACT GCC CCG GAC G}3'
NvFGFBP1 (F) – 5' {GCG TTC GAA GGG TGG GGG TG}3'
NvFGFBP1 (R) – 5' {GGG ACA GCT TCG GGT GCG TG}3'
NvGAPDH (F) – 5' {TGA CAA CTT CCA CAT CGT CGA GGG T}3'
NvGAPDH (R) – 5' {GAG TGC AAT ACC GGC ACC AGC}3'
NvG-TypeLysozyme (F) – 5' {CCA CAA GAT CAG AGG TGC TTG GAA C}3'
NvG-TypeLysozyme (R) – 5' {CCT CCA GTG GTG CGG CTG TC}3'
NvH3F3B (F) – 5' {CTG CCA GGA AAA GCG CGC CA}3'
NvH3F3B (R) – 5' {CTT GCC TCC TGC AGA GCC CC}3'
NvHBA (F) – 5' {GCT CAG TGA CAG GCA CGC CC}3'
NvHBA (R) – 5' {TGT GGT GTT TTG GTA TGG AGT GGC A}3'
NvHSP40 (F) – 5' {ACA CTG CAC TCA ACT GCA TGG C}3'
NvHSP40 (R) – 5' {GCA CGG AGA CAC ACA GTG CGG}3'
NvHSP70 (F) – 5' {GGA CGA CAC TTT GTC GCG CTG}3'
NvHSP70 (R) – 5' {ACC AGC ACC TCG TGG CGT TC}3'
NvJunD (F) – 5' {CCA ACT CAG GCG AGG CCA GC}3'
NvJunD (R) – 5' {CCA GAG CCA CCC TCC CCT CC}3'
NvKeratin17 (F) – 5' {CGT TCC GGA GCT CGG TCA GC}3'
NvKeratin17 (R) – 5' {AAC GTG GAA GTG GAC GCC GC}3'
NvKeratinTypeI (F) – 5' {TCT CCA GCG CTT CCA CCA GC}3'
NvKeratinTypeI (R) – 5' {TCA TGG GCG CCA CGA TCG AC}3'
NvMMP3/10a (F) – 5' {TCG GCC GCA TCA GCA ACT CC}3'
NvMMP3/10a (R) – 5' {TCG CTG CTG CTG CTC CTG TG}3'
NvMYND (F) – 5' {GTT TGC TGG GCT CTC TGG CA}3'
NvMYND (R) – 5' {GCT GTG CAA TGG CCC GAC AAG}3'
NvNRD1 (F) – 5' {TGT CAG GCC CTC TAT GAA ACG CA}3'
NvNRD1 (R) – 5' {TGC GAA CCA AGC AAA CCC TCG}3'
NvPGLYRP1 (F) – 5' {ACG AAA CCC GAA CCA GGC CG}3'
NvPGLYRP1 (R) – 5' {CCA GTC CTC GTG AAG CGC CC}3'
NvPTMA (F) – 5' {AGC ACA TGC CTT ACA TTT CGG TGC}3'
NvPTMA (R) – 5' {ACG CAC ACT TTG ACA GCA CAC AG}3'
NvRAD51 (F) – 5' {TCG GCA GAT GGG GCA TTC GC}3'
NvRAD51 (R) – 5' {GAG GCC TTA ACG GTG CGC CA}3'
NvRPL37 (F) – 5' {TGG CCG TAT GAG GCA CTT GAA GGT}3'
NvRPL37 (R) – 5' {GGA TGC TGC AAC TGC CGC CC}3'
NvRPS27A (F) – 5' {AAG TGC GTC CGT CTT CCA GC}3'
NvRPS27A (R) – 5' {CCG CCA TTG GGA GGA GGT GG}3'
NvRUFY1 (F) – 5' {CTC GTG TTG CAA CTG CTG CCG}3'
NvRUFY1 (R) – 5' {AGG AGC AGT GAC CGT GGG GC}3'
NvSec61 (F) – 5' {CGC GGG GAG GTT GAG CGT C}3'
NvSec61 (R) – 5' {AGC CGA TGT AGT TCG GGC AGC}3'

NvTβ4 (F) – 5' {ACA CGG GGT CTG ACA AGC CTG A}3'
NvTβ4 (R) – 5' {TGC AGG CCT CCT TCT CCT GT}3'
NvTβ10/15 (F) – 5' {GCA GAC CTC TCT TTG GTG GA}3'
NvTβ10/15 (R) – 5' {GTC CGT CAC TAA CCA AAC AC }3'
NvTIMP1 (F) – 5' {CCT ACC CCC TCA CTG CCC TGG}3'
NvTIMP1 (R) – 5' {CAG GCT GCG GCT GTG AGA TCG}3'
NvTXNDC9 (F) – 5' {GGC CCA GAG ATG GTG GCT CC}3'
NvTXNDC9 (R) – 5' {GCC CCA TTC CTA ACT GAG CGG C}3'
NvWntless (F) – 5' {GCG TTC GTA GGC CCT GGA GC}3'
NvWntless (R) – 5' {CAG GAG TTC CCG CAG CAG CG}3'

RACE Adapter Primers

RACE Adapter Primer (AP) – 5' {GAC TCG AGT CGA CAT CG}3'
RACE dT₁₇ Adapter Primer (dT₁₇ AP) – 5' {GAC TCG AGT CGA CAT CGA TTT TTT TTT TTT TTT TT}3'

RACE Gene Specific Primers

βcatenin 3' Outer – 5' {AAC CCA AGC TGA TTT GAT GG}3'
βcatenin 3' Inner – 5' {AAC CCC GAA GAG GAA GAT GT}3'
βcatenin 5' Outer – 5' {ATCAAATCAGCTTGGGTTGC}3'
βcatenin 5' Inner – 5' {GCAGCTTTTCGATCTGGTTTC}3'
Dapper 3' Outer – 5' {CCA ACT GTT TTG GGG ACA GT}3'
Dapper 3' Inner – 5' {AAG GCT TCG CAT AAC CTC AA}3'
Dapper 5' Outer – 5' {GAG GTT ATG CGA AGC CTT GA}3'
Dapper 5' Inner – 5' {GCG GTT TGC ATA GGA AGT GT}3'
Tβ 5' RACE – 5' {ATG GCN GAY AAR CCN GAY}3'
Tβ4 3' Outer – 5' {GGA CAC TCA CGC TCT GTC AA}3'
Tβ4 3' Inner – 5' {GAA GGC AAT GCT TGT GGA AT}3'
Tβ10/15 3' Outer – 5' {GAT TTC TTG CAG GCC TCC TT}3'
Tβ10/15 3' Inner – 5' {AAA GTT CTC GAC CTC CGA CA}3'
Wntless 3' Outer – 5' {TGT GGC AAT TGT AAG GGA CA}3'
Wntless 3' Inner – 5' {TCT GGT GGG AGG CTT AAT TG}3'
Wntless 5' Outer – 5' {GGA ACC AGG GAG TCA TTT CA}3'
Wntless 5' Inner – 5' {CCC AAG GCA TAA ACC AGC TA}3'

Appendix III – Solution Recipes

Amphibian Differentiation Media (ADM)

Add to 339ml of Minimum Essential Medium (MEM; Gibco, USA):

- 144ml sterile dH₂O
- 2.5ml heat inactivated fetal bovine serum (FBS; HyClone, USA)
- 5ml penicillin/streptomycin/fungizone (Gibco, USA)
- 5ml L-glutamine (Gibco, USA)
- 5ml insulin* (Sigma, USA)

Store at 4°C.

* For insulin stock, dissolve 50mg insulin in 5ml of 0.1M hydrochloric acid (HCl; Fisher, USA). Dilute by slowly adding 45ml of 80% PBS (diluted Dulbecco's Modified PBS; HyClone, USA) and shake slightly. Filter sterilize through a 0.2µm syringe filter, aliquot, and store at -20°C.

Amphibian Growth Media (AGM)

Add to 315ml of Minimum Essential Medium (MEM; Gibco, USA):

- 120ml sterile dH₂O
- 50ml heat inactivated fetal bovine serum (FBS; HyClone, USA)
- 5ml penicillin/streptomycin/fungizone (Gibco, USA)
- 5ml L-glutamine (Gibco, USA)
- 5ml insulin* (Sigma, USA)

Store at 4°C.

* For insulin stock, dissolve 50mg insulin in 5ml of 0.1M hydrochloric acid (HCl; Fisher, USA). Dilute by slowly adding 45ml of 80% PBS (diluted Dulbecco's Modified PBS; HyClone, USA) and shake slightly. Filter sterilize through a 0.2µm syringe filter and store at -20°C.

Antifade

- 50:50 TBS:glycerol (Fisher, USA)
- 1% N-propyl gallate (Sigma, USA)

Heat to 65°C to dissolve and store at 4°C, protected from light.

Blocking Solution (for ISH)

5ml sheep serum (Sigma, USA)
5ml 10% Blocking Reagent (Roche, Germany)
5ml 5x MABT
10ml dH₂O

EDTA-Based Decalcification Solution

Add to 75ml dH₂O:

14g EDTA (BioShop, Canada)
9ml of 30% ammonium hydroxide (Fisher, USA)

Adjust pH to 7.4 with hydrochloric acid (HCl; Fisher, USA) and bring total volume to 100ml with dH₂O.

Eosin Y

Stock solution:

1g eosin Y (Sigma, USA)
200mL dH₂O

Working solution:

0.5ml glacial acetic acid (Fisher, USA)
100ml eosin Y stock solution

Ethanol:Glycine Fixative

Add to 250ml dH₂O:

0.94g glycine (50mM final; EMD, USA)

Adjust pH to 2.0 with hydrochloric acid (HCl; Fisher, USA) and filter through 0.45µm bottle-top filter. Store at 4°C.

Combine 70ml of 100% ethanol (Commercial Alcohols, Canada) with 30ml of 50mM glycine and store at -20°C.

Hybridization Buffer (for ISH)

Use RNase-free water for all solutions:

- 1ml 10x salt solution*
- 5ml deionized formamide (Invitrogen, USA)
- 2ml 50% dextran sulfate (Sigma, USA)
- 1ml 10mg/ml rRNA (Invitrogen, USA)
- 100µl 100x Denhardt's solution†
- 900µl dH₂O

Store in aliquots at -20°C.

†*Denhardt's Solution*

- 2g BSA (Sigma, USA)
- 2g Ficoll 400 (Sigma, USA)
- 2g PVP (polyvinyl pyrrolidone; Sigma, USA)

Bring total volume to 100ml with dH₂O. Store in aliquots at -20°C.

**Salt Solution (10x)*

- 114g sodium chloride (NaCl; Sigma, USA)
- 14.04g tris-HCl (pH 7.5; Fisher, USA)
- 1.34g tris base (Fisher, USA)
- 7.8g sodium dihydrogen phosphate dihydrate (NaH₂PO₄·2H₂O; EMD, USA)
- 7.1g disodium hydrogen phosphate (Na₂HPO₄; Sigma, USA)
- 100ml 0.5M EDTA (BioShop, Canada)

Bring total volume to 1L with dH₂O and autoclave.

Luria Broth/Agar (LB Media/Plates)

Add to 900ml dH₂O:

10g tryptone (Sigma, USA)
5g yeast extract (BD, USA)
5g sodium chloride (NaCl; Sigma, USA)

For Agar, add 15g BactoAgar (BD, USA).

Adjust pH to 7.0 with sodium hydroxide (NaOH; Fisher, USA) and bring total volume to 1L with dH₂O. Autoclave to sterilize. For agar plates, add appropriate antibiotic when cooled to <50°C [Ampicillin – 50ug/ml final (Sigma, USA); Kanamycin – 20ug/ml final (Sigma, USA)]. Pour into sterile plates and store at 4°C.

MABT (5x; for ISH)

Add to 500ml dH₂O:

58.05g maleic acid (Sigma, USA)
32g sodium hydroxide (NaOH; Fisher, USA)
43.8g sodium chloride (NaCl; Sigma, USA)
5ml Tween-20 (EMD, USA)

Dissolve maleic acid first and add NaOH to correct pH before adding the other reagents.

Mammalian Differentiation Media (DM)

Add to 475ml of Dulbecco's Modified Eagle Medium (HyClone, Gibco, USA):

10ml heat inactivated horse serum (HS; Gibco, USA)
5ml penicillin/streptomycin (Gibco, USA)
5ml L-glutamine (Gibco, USA)
5ml sodium pyruvate (Sigma, USA)

Store at 4°C.

Mammalian Growth Media (GM)

Add to 435ml of Dulbecco's Modified Eagle Medium (DMEM, HyClone, USA):

50ml heat inactivated fetal bovine serum (FBS; HyClone, USA)
5ml penicillin/streptomycin (Gibco, USA)
5ml L-glutamine (Gibco, USA)
5ml sodium pyruvate (Sigma, USA)

Store at 4°C.

MS-222 (0.05%)

Add to 1L dechlorinated H₂O:

0.5g 3-aminobenzoic acid ethyl ester (Sigma, USA)
0.6g sodium hydrogen carbonate (NaHCO₃; VWR, USA)

Make fresh before use.

Phosphate Buffered Saline (PBS, 10x)

(Also: Phosphate Buffered Saline/Tween-20 - PBST)

Add to 800mL dH₂O:

4.26g sodium phosphate dibasic (Na₂HPO₄-7H₂O; VWR, USA)
1.44g potassium hydrodgen phosphate (KH₂PO₄; Sigma, USA)
90g sodium chloride (NaCl; Sigma, USA)

Adjust pH to 7.4 with sodium hydroxide (NaOH; Fisher, USA) or hydrochloric acid (HCl; Fisher, USA) and bring total volume to 1L with dH₂O. Aliquot to 1X; to make PBST, add 500µl Tween-20 (EMD, USA) per 1L PBS.

RIPA Lysis Buffer

Add to 150ml dH₂O:

10ml 1M tris-HCl (pH 7.4; Fisher, USA)
6ml 5M sodium chloride (NaCl; Sigma, USA)
400µl 0.5M EDTA (BioShop, Canada)
2mL nonidet P-40 (EMD, USA)
0.5g deoxycholic acid (Sigma, USA)

Bring total volume to 200ml with dH₂O. Before use, add one protease inhibitor tablet [Complete, Mini, EDTA-free Protease Inhibitor Cocktail Tablets (Roche, Germany)] to 7ml of buffer.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) Gels

4% Separating Gel

Add to 3.075ml dH₂O:

1.25ml 0.5M tris-HCl (pH 6.8; Fisher, USA)
25µl 20% SDS (Sigma, USA)
670µl acrylamide/bis-acrylamide (30%/0.8% w/v; National Diagnostics, USA)
50µl ammonium persulfate (10% w/v; BioRad, USA)
10µl TEMED (Calbiochem, USA)

10% Stacking Gel

Add to 4.92ml dH₂O:

3ml 1.5M tris-HCl (pH 8.8; Fisher, USA)
60µl 20% SDS (Sigma, USA)
3.96ml acrylamide/bis-acrylamide (30%/0.8% w/v; National Diagnostics, USA)
120µl ammonium persulfate (10% w/v, BioRad, USA)
12µl TEMED (Calbiochem, USA)

Staining Buffer (for ISH)

10ml 0.1M tris-HCl (pH 9.5; Fisher, USA)/0.1M sodium chloride (NaCl; Sigma, USA)

Add 200µl of NBT/BCIP Stock Solution (Roche, Germany) to 10ml of staining buffer DIG system applications.

Tris-Buffered Saline (TBS)

Add to 800mL of dH₂O:

6.05g tris base (Fisher, USA)
8.76g sodium chloride (NaCl; Sigma, USA)

Adjust pH to 7.5 with hydrochloric acid (HCl; Fisher, USA) and bring total volume to 1L with dH₂O.

Wash Buffer (for ISH)

Make fresh before use:

25ml of 20x SSC
250ml deionized formamide (Invitrogen, USA)
500µl Tween-20 (EMD, USA)
225ml dH₂O

SSC (20x)

Add to 500ml of dH₂O:
175.3g sodium chloride (NaCl; Sigma, USA)
88.2g sodium citrate (Sigma, USA)

Adjust pH to 7.0 and bring total volume to 1L.