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Gene Regulatory Pathways Driving Central Nervous System Regeneration in Zebrafish

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GENE REGULATORY PATHWAYS DRIVING CENTRAL NERVOUS SYSTEM REGENERATION IN ZEBRAFISH

by

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ABSTRACT
GENE REGULATORY PATHWAYS DRIVING CENTRAL NERVOUS SYSTEM REGENERATION IN ZEBRAFISH

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Damage to the central nervous system (CNS) circuitry of adult mammals results in permanent disability. In contrast, the ability to regenerate damaged CNS nerves and achieve functional recovery occurs naturally in fish. The ability of fish to successfully regrow damaged CNS nerves is in part a consequence of their ability to re-express key neuronal growth-associated genes/proteins in response to CNS injury. On such protein is Growth-Associated Protein-43 (Gap43), a protein which is highly enriched in axonal growth cones during CNS development and regeneration. Experiments conducted in mammals have demonstrated that ectopic expression of GAP-43 improves axonal re-growth after injury. Using zebrafish optic nerve as a model for successful CNS regeneration, we have identified that re-expression of the *gap43* gene is crucial for regenerative axon growth *in vivo*. Using a combination of *in vivo* reporter assays and *in vivo* regeneration assays, we also identified transcriptional regulatory pathways that are essential for *gap43* gene expression in regenerating CNS neurons. We found that transcription factors Ascl1a, Atf3, cJun and Stat3 are required for both re-

initiating *gap43* expression and driving axon outgrowth in response to optic nerve injury. Futhermore, our results indicate that transcription factors Ascl1a, Atf3 and cJun function cooperatively to re-initiate *gap43* expression in a manner that is conserved across highly divergent teleost species. Together, these experiments provide insights into regulatory mechanisms driving successful CNS regeneration, thereby revealing potential targets that may be manipulated to improve regenerative ability in mammals.

To Amma, Appa and Chikku

For your unconditional love, support and faith in me, that made this possible

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Akt	serine/Threonine-specific protein kinase
AP-1	activator protein-1
Ascl1a	achaete-scute family basic helix-loop-helix transcription factor 1a
ATF3	activating transcription factor 3
BDNF	brain-derived neurotrophic factor
bHLH	basic helix-loop-helix
bp	base pair
bZIP	basic leucine zipper domain
C/EBP	CCAAT/enhancer binding protein
Ca2+	calcium
cAMP	cyclic adenosine monophosphate
CAP-23	cytoskeletal associated protein 23
CBP	CREB binding protein
CD44	cluster of differentiation 44
cDNA	complementary deoxyribonucleic acid
CGRP	calcitonin gene-related peptide
ChIP	chromatin immunoprecipitation
CNS	central nervous system
CNTF	ciliary neurotrophic factor
CREB	cyclic adenosine monophosphate response element binding protein
CSPG	chondroitin sulfate proteoglycans

CST	cortico-spinal tract
Ct	cycle threshold
DAPI	4',6-diamidino-2-phenylindole
DINE	damage induced neuronal endopeptidase
DNA	deoxyribonucleic acid
dpi	days post injury
Ef1α	elongation factor 1 alpha
ERK	extracellular regulated kinase
FOS	finkel osteogenic sarcoma
GAP- 43	growth associated protein-43
GFP	green fluorescent protein
Hsp27	heat shock protein 27
HuC	Hu-antigen C
IACUC	institutional animal care and use committee
IEG	immediate early gene
IL-6	interleukin-6
JAK	janus activated kinase
JNK	c-jun NH2-terminal kinase
KLF	kruppel-like factors
LIF	leukemia inhibitory factor
LINGO-1	leucine rich repeat and immunoglobulin domain containing 1
MAG	myelin associated glycoprotein
MAPK	mitogen activated protein kinase
MARCKS	myristoylated alanine-rich protein kinase C substrate
MASH1	mammalian achaete scute homolog 1

MO	morpholino
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
NCS	normal calf serum
Nex1/MATH2	novel brain specific helix-loop-helix protein
NgR	reticulon 4 receptor
NOGO	reticulon 4
Omgp	oligodendrocyte myelin glycoprotein
p38 MAPK	p38 mitogen-activated protein kinases
p53	tumor suppressor protein 53
Pax6a	paired box 6a
PBST	tween 20 in phosphate buffered saline
PC12	pheochromocytoma cell line 12
PCAF	p300/CREB binding protein-associated factor
PFA	paraformaldehyde
PI3K	phosphoinositide 3-kinase
PIP2	phosphatidylinositol 4,5-bisphosphate
PKC	protein kinase C
PNS	peripheral nervous system
qPCR	quantitative polymerase chain reaction
Rab12	ras-related protein 12
RAG	regeneration associated gene
RGC	retinal ganglion cell
RNA	ribonucleic acid
ROCK	Rho-associated protein kinase

S42A	serine 42 substitution to alanine
SC	schwann cell
Seq	sequencing
Sfpq	splicing factor proline/glutamine-rich
SOCS3	suppresor of cytokine signaling 3
Sox11	(sex determining region-Y)-box 11
Sp1	specificity protein 1
SPRR1a	small proline-rich protein 1A
STAT3	signal transducer and activator of transcription factor 3
TFs	transcription factors
Tris	tris(hydroxymethyl)aminomethane hydrochloride
Tuba1a	tubulin, alpha 1a
WT	wild type

Chapter I

Introduction

The nervous system is a highly specialized organ system that synchronizes the human body's responses to external and internal stimuli. The nervous system is broadly classified into the central nervous system (CNS) composed of the brain, spinal cord and retina and the peripheral nervous system (PNS) comprised of nerves and ganglia innervating the periphery. Everything we do from learning new things, recollecting old memories, as well as perceiving and responding to our environment is dependent on the nervous system. Problems affecting the nervous system can therefore be severely debilitating, causing a spectrum of defects including loss of memory, motor control, and sensory perception among others. While humans have the ability to respond to peripheral nerve injury through successful regeneration and recovery of function, a similar response is not mounted following insult to the CNS. Rigorous scientific efforts have been long underway to find strategies to limit or reverse the damage caused due to CNS injuries. One way to approach this problem has been to understand mechanisms underlying successful regeneration in a variety of experimental models in the hope of re-capitulating similar mechanisms to improve regeneration in humans.

In order to understand the mechanisms underlying successful CNS regeneration, two common approaches are taken. The first approach has been to

understand mechanisms underlying peripheral regeneration. The other approach has been to utilize animal models that are intrinsically capable of mounting a successful regenerative response following CNS injury. We have taken the second approach by studying zebrafish optic nerve regeneration to identify gene regulatory networks underlying successful vertebrate CNS regeneration.

Barriers to regeneration in the mammalian CNS

It is well accepted that there are two main barriers to regeneration in the adult mammalian CNS: 1) An inhibitory external environment that hinders axon re-growth 2) Failure to initiate a transcriptional program conducive of axon outgrowth in response to injury.

Inhibitory external cues

The earliest suggestion that the environment surrounding injured axons in the mammalian CNS was inhibitory, comes from the landmark histological studies carried out by Ramon ý Cajal (1929). Advances in understanding the inhibitory environment have revealed broadly two broad classes of inhibition: 1) the inhibitory molecules produced by the myelinating glia of the CNS (oligodendrocytes) and 2) the formation of a glial scar initiated by reactive astrocytes. Together these form both physical and chemical barriers that obstruct CNS regeneration.

Myelin is the insulation wrapped around an axon for efficient propagation of electrical signaling along the axon. After CNS injury, myelin debris becomes dispersed throughout the injury site, forming physical barriers that prevent axons

from re-connecting to their targets. In addition, many classes of inhibitory molecules are produced by oligodendrocytes including chondroitin sulfate proteoglycans (CSPGs), semaphorins, NogoA, myelin-associated glycoprotein (MAG) and oligodendrocyte-myelin glycoprotein (Omgp) (reviewed by Sharma *et al*., 2012, Pasterkamp and Verhaagen, 2006, Schwab 2004, Filbin 2003). All these inhibitory molecules signal through a receptor complex comprised of NgR, p75NTR and LINGO-1 (Park et al., 2005; Shao et al., 2005).

In addition to the myelin associated inhibitory molecules, the other major barrier to axon re-growth is the formation of a glial scar. The glial scar is the result of accumulation of microglia, oligodendrocytes and astrocytes at the site of injury (Rudge *et al*., 1990). The inhibition from the scar is both physical and chemical in nature. Inhibitory cues found in the reactive glial scar include aforementioned CSPGs which restricts neural repair by inhibiting axon extension across the lesion and by inhibiting collateral sprouting by spared axons near the lesion (reviewed by Sharma *et al*., 2012). Repressive axon guidance cues expressed during development such as ephrins and semaphorins also limit axonal regeneration in the injured mammalian CNS (Kaneko et al., 2006; Kantor et al., 2004; Pasterkamp and Verhaagen, 2006).

Improving regenerative ability through the manipulation of extrinsic inhibitory cues has been met with limited success. Several studies have been conducted with mice that genetically lack inhibitory molecules or receptors to determine if such manipulations enhance CNS regeneration. NogoA knockout mice display little to no improvement in regeneration after spinal cord injury (Kim

et al., 2003, Simonen *et al*., 2003, Zheng *et al*., 2005). Mice lacking the Nogo receptor, NgR also show little increase in regeneration following corticospinal axonal injury (Zheng *et al*., 2005). Mice lacking MAG display very modest enhancement in axon regeneration following spinal cord injury (Li *et al*., 1996). Recently, it was observed that triple mutant mice lacking Nogo, MAG and OMgp showed little improvement in regeneration of corticospinal or raphesinal axons following spinal cord injury (Lee *et* al., 2010). Inhibiting the action of molecules that form the reactive glial scar through enzymatic degradation or using antibodies to neutralize such molecules also improves regeneration but to a very limited extent. (Moon and Fawcett 2001; Tan *et al*., 2006). Together these studies suggest that simply removing the inhibitory environment alone is insufficient to promote robust axon re-growth and that improving the neuron-intrinsic capacity for axon regrowth is necessary for successful regeneration.

Intrinsic growth capacity for axon regeneration

Neutralizing the external inhibitory environment still leads to only a small percentage of injured axons showing successful re-growth following CNS injury. Hence studies focusing on manipulating the intrinsic growth capacity of neurons to induce axon re-growth are on the steady rise. In response to axonal injury, three major molecular events occur that ultimately determine the intrinsic ability to mount a successful regenerative response (summarized in Fig 1). The signaling cascades ensuing axonal injury have been the focus of several studies and findings that have revealed the importance of these pathways in successful regeneration are broadly discussed in the next section.

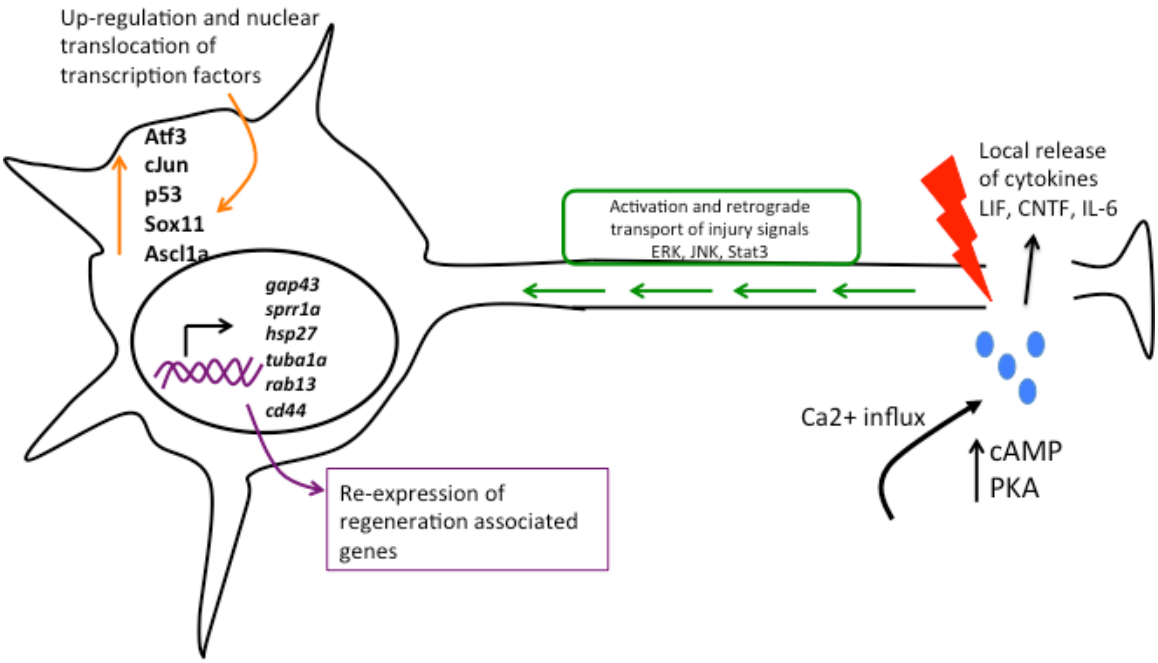


Fig. 1. Signal transduction in response to axonal injury. Axon injury leads to a cascade of signaling events initiating with an increase in intracellular calcium that corresponds to an increase in levels of cAMP and PKA. Local release of cytokines in the injury site also stimulates the activation and retrograde transport of injury derived signals such as ERK, JNK and Stat3. Retrograde delivery of injury derived signals initiates upregulation of critical transcription factors associated with axonal injury such as Atf3, cJun, Ascl1a, p53, Sox11 etc. Transcription factors translocate to the nucleus and along with co-activators re-initiate transcription of critical regeneration associated genes such as *gap43, sprr1a*, *hsp27* and *tuba1a* among others.

1) Retrograde transport of injury signals to the neuronal cell body

Activation of pro-regenerative genes is induced by retrograde signaling pathways generated at the stump of the lesion that are transported back to the neuronal cell body within 12-24 hours following injury (Hanz *et al.*, 2003; Ben-Yaakov *et al.*, 2012). Cytokines such as leukemia inhibitory factor (LIF), interleukin-6 (IL-6) and ciliary neurotrophic factor (CNTF) are released locally at the sites of peripheral lesion and contribute to the activation of a successful regenerative program through activation of intracellular JAK/STAT signaling pathways (Cao *et al.*, 2006, Sendtner *et al.*, 1992, Subang and Richardson, 2001). Deletion of suppressor of cytokine signaling-3 (SOCS3), a negative regulator of JAK/STAT signaling pathways, greatly enhances axon regeneration after optic nerve injury in mammals, through induction and maintenance of a repertoire of regeneration-associated genes, all of which are crucial for functional regeneration (Sun *et al.*, 2011).

The other class of injury-related signals are initiated in response to an intracellular rise in calcium and cAMP due to a rapid depolarization following axonal injury. These molecules include the mitogen-activated protein kinases (MAPK), extracellular signal-regulated kinase (ERK), p38, c-Jun N-terminal kinase (JNK), Phosphotidylinositol 3 kinase (PI3K) and mammalian target of rapamycin (mTOR) among others (reviewed by Patodia and Raivich, 2012). Deletion of phosphatase and tensin homolog (PTEN), a negative regulator of mTOR, enhances retinal ganglion cell (RGC) axon regeneration in response to optic nerve injury in mammals. Notably, co-deletion of PTEN and SOCS3 leads

to a synergistic effect with increased RGC axon regeneration in response to injury (Sun *et al*., 2011). Therefore, retrograde injury signals are crucial in initiating a successful regenerative program and are attractive targets that can be manipulated to improve CNS regeneration in mammalian neurons.

2) Upregulation and activation of regeneration-associated transcription factors

The retrograde transport of key injury signals mediate the rapid activation and nuclear translocation of a suite of regeneration-associated transcription factors (Fig 1). This injury-induced transcription is critical in gaining growth competence following injury (Smith and Skene, 1997). Phosphoproteomic and microarray studies have identified nearly 400 redundant axonal signaling networks connected to 39 transcription factors implicated in the sensory neuron response to axonal injury (Michaelevski *et al*., 2010, reviewed by Patodia and Raivich, 2012). These transcription factors include cJun, JunD, Activating transcription factor-3 (Atf3), cyclic AMP response binding element (CREB), signal transducer and activator of transcription 3 (Stat3), CCAAT/ enhancer binding proteins (C/ EBPs), p53, Kruppel like factors (Klfs) and Sox11 among others (reviewed by Patodia and Raivich, 2012). Once activated, these transcription factors bind to specific promoter regions resulting in transcriptional activation or repression of a wide variety of target genes that is critical in initiating and sustaining a successful regenerative response.

Most regeneration associated transcription factors are upregulated in response to peripheral nerve injuries in mammals, but not in response to CNS injuries. In principle, two approaches have been utilized in understanding transcriptional

regulation of axon growth. The first approach involves knockdown of transcription factors in neurons capable of functional regeneration to identify specific transcription factors necessary for successful axon re-growth. The second approach involves forced expression of transcription factors in neurons incapable of functional regeneration; thereby testing whether the ectopic expression is sufficient to induce re-growth in injured mammalian CNS axons. Both approaches have been successful in identifying transcriptional complexes involved in nervous system regeneration leading to a better understanding of transcriptional regulatory pathways governing successful regeneration (reviewed by Tedeschi *et al*., 2012, Patodia and Raivich *et al*., 2012). The functional roles of select transcription factors during nervous system regeneration are summarized in Table 1. Since a single transcription factor likely regulates several regeneration- associated genes, they are appealing targets to identify gene regulatory networks underlying successful regeneration. Designing therapeutic interventions centered around transcription factors are also likely to be effective, since delivery of a single transcription factor may lead to the activation of multiple downstream regeneration associated genes, thereby enhancing the intrinsic ability to initiate a regenerative response. The orchestrated expression of the downstream regeneration-associated genes ultimately determines regenerative success.

3) Re-expression of crucial regeneration-associated genes

The injury-induced transcription factors regulate the expression of a set of genes involved in cell-cell signaling, axon outgrowth and cell survival, collectively referred to as regeneration-associated genes (RAGs). RAGs have been

identified in two classes of neurons showing successful regeneration - mammalian models of PNS regeneration and fish models of CNS regeneration. DNA microarray experiments using aforementioned models have identified several RAGs (Bonilla *et al.,* 2002, Costigan *et al*., 2002, Tanabe *et al*., 2007, Boeshore *et al.,* 2004, Nilsson *et al*., 2005, Veldman *et al*., 2007). These genes are diverse in function, but ultimately most of the functions are related to the cell membrane and the cytoskeleton.

One class of RAGs encodes neuronal growth-associated proteins that function as cytoskeletal adaptors by modulating cytoskeletal dynamics during axon outgrowth, guidance and regeneration. Growth cones found at the tips of growing axons are responsible for modulating axon growth, guidance and synaptic target recognition through modulation of cytoskeletal dynamics. For successful regeneration to occur, gene expression must synchronize with cytoskeletal dynamics to efficiently distribute signaling components and re-assemble structural components (Hur *et al*., 2012). The molecules that modulate remodeling of the cytoskeleton and the reformation of the growth cone after nerve injury are of critical importance to regenerative success. This is because these molecules directly affect growth cone response to inhibitory molecules, the speed and extent of axon regeneration, and target innervation (Hur *et al*., 2012).

Growth associated protein-43 (Gap43) and cytoskeleton-associated protein-23 (CAP-23) are members of an important family of cytoskeletal adaptor molecules (Skene and Willard 1981, Verhaagen *et al*., 1986, Frey *et al*., 2000, Bomze *et al*., 2001, Zhang *et al* ., 2005, Patodia and Raivich 2012). Gap43 and

Cap23 were found highly enriched in growth cones of neurons during both development and regeneration (Skene *et al.,* 1989, Goslin and Banker 1990, Bomze *et al*., 2001). Gap43 and Cap23 modify actin filament polymerization, organization and disassembly, through interactions with phospholipids such as PIP2, calcium/calmodulin and protein kinase C (PKC) to elicit growth cone activity (Skene 1990, Ide 1997, Laux *et al.,* 2000, Henley and Poo 2004, Kulbatski *et* al.,2004).

Expression of Gap43 is downregulated in most mature neurons and re-initiated in response to injury in neuronal populations capable of mounting a successful regenerative response (Bormann *et* al., 1988, Kaneda *et* al., 2008, Bomze *et* al., 2001). The primary sensory neurons from dorsal root ganglia (DRGs) has two major branches, a peripheral axon that innervates peripheral targets and a central axon that relays the information to the spinal cord. These two branches of DRG axons have differential capacities for axon regeneration following injury. While the peripheral axon regenerates successfully after lesion, the injury of the central branch from the same DRG neuron fails to elicit a successful response. However, lesioning the peripheral branch prior to injuring the central branch, greatly improves regeneration of both the central and peripheral branches, in what has been termed as a conditioning effect (Liu *et al*., 2011). Differences in ability to initiate a gene program supportive of axon growth are thought to underlie the differential capacity for regeneration in the peripheral versus central branch of DRG neurons. Consistent with this hypothesis, Gap43 is upregulated in response to a peripheral lesion in DRGs, but not in response to a

lesion to the central branch of the DRG (Schreyer and Skene, 1993). However, treatment with cyclic AMP (cAMP) and a conditioning lesion to the DRG, both result in expression of Gap43 in the central branch of injured DRGs (Qiu *et al*., 2002). Optic nerve injury affects only a single class of neurons - RGCs. In zebrafish, RGCs have a high capacity for regenerative growth and display increased levels of Gap43 throughout the regenerative process (Kaneda *et al*., 2008). However, the spinal cord of zebrafish has several types of neurons with varying regenerative capacities (Becker *et al*., 1998). It is observed that the differing capacity for regeneration in these neurons is directly correlated with their ability to re-express crucial regeneration associated genes such as Gap43 and cell adhesion protein L1 (Becker *et al*., 2005). Thus re-expression of Gap43 is correlated with successful intrinsic ability to initiate and sustain a regenerative response.

While Gap43 expression is not re-initiated in mammalian CNS neurons, studies have demonstrated that ectopic expression of Gap43 improves CNS regeneration in injured adult mammalian neurons. Co-expression of Gap43 and Cap23 in DRG neurons, leads to a 60-fold increase in the re-growth of injured dorsal column axons (Bomze *et al*., 2001). Constitutive over-expression of Gap43 in combination with cell adhesion protein L1 leads to increased axon regeneration of cerebellar purkinje axons *in vivo* (Zhang *et al*., 2005). Since artificial expression of Gap43 improves regeneration outcomes in injured adult mammalian CNS neurons, it is important to understand how Gap43 expression is re-initiated in neurons capable of functional recovery.

Transcriptional regulation of Gap43

In vitro analysis of the mammalian gap43 gene promoter

The mammalian *gap43* gene promoter consists of both activators and repressors responsible for directing spatial and temporal expression of the *gap43* gene. Two closely related promoters with multiple transcription start sites have been identified for mammalian Gap43. There is a TATA-less promoter sequence proximal to the protein-coding region and a more distal promoter that contains consensus TATA box sequences (Ortoft *et al*., 1993, Eggen *et al*., 1994, Starr *et al*., 1994, Nedivi *et al*., 1992). *In vitro* promoter analysis identified a 386 bp region that was sufficient to drive neural specific *gap43* expression (Nedivi *et al*., 1992). *In vivo*, however a 1.6 kilobase promoter fragment was required for neural specific expression of *gap43* in transgenic mice (Vanselow *et al*., 1994). Within this promoter region, two key *cis*-acting elements were identified. The first is an E-box binding site, which depending on interactions with members of basic helix loop helix (bHLH) family of transcription factors, could direct either transcriptional activation or repression (Chiaramello *et al*., 1996). Transcription factor Nex1/ MATH-2 belongs to the bHLH family of proteins and was capable of activating the *gap43* promoter *in vitro* (Shimizu *et al*., 1995, Uittenbogaard *et al*., 2003). The second regulatory element identified was an AP-1 element (Weber and Skene, 1998). Transcription factors cJun and cFos are capable of binding to the AP-1 element as homodimers or heterodimers. Transcription factor cJun is well documented in driving axon outgrowth in the PNS (Raivich *et al*., 2004). It is not

known whether this AP-1 element through cJun regulates *gap43* expression. Presence of *cis*-acting elements capable of binding to members from different transcription factor families hint that expression of *gap43* is likely regulated by multiple transcription factors.

Analysis of the *gap43* promoter has revealed differences in function and conservation of promoter regions across species (Udvadia *et al.,* 2001, Udvadia *et al.,* 2008, Kusik *et al*., 2010). For instance, rat *gap43* promoter sequences were able to direct transgene expression in zebrafish during nervous system development (Reinhard et al., 1994) but not during optic nerve regeneration (Udvadia *et al*., 2001). These results indicated that promoter regions responsible for directing *gap43* expression during regeneration have diverged between species and that regulatory pathways governing nervous system regeneration are different from pathways modulating development (Udvadia *et al*., 2001). Teleost fish possess distinct advantages that have led to their use as models to identify promoter elements underlying successful re-expression of growth associated genes and axon outgrowth.

Teleost models of transcriptional regulation and CNS axon outgrowth

Teleost fish successfully re-express growth-associated genes in response to CNS injury resulting in functional recovery. In addition to upregulating positive regulators of axon growth in response to CNS injury, fish also upregulate many of the same negative modulators of axon regeneration observed in mammals, such as SOCS3 and Sfpq. However, in contrast to mammals, fish are able to overcome these inhibitory cues and mount a successful regenerative response

(Elsaedi *et al*., 2014). This finding demonstrates that the barriers to regeneration elicted by CNS nerve injury are similar in both fish and mammals. Thus it is the intrinsic ability to mount a successful response, which differs and ultimately determines regenerative success. The above mentioned factors along with the relative ease in creating mutant transgenic lines and delivering knockdown reagents, have rendered teleost fish as ideal models to dissect regulatory pathways governing re-expression of crucial regeneration associated genes and axon outgrowth (Udvadia *et al*., 2008, Kusik *et al*., 2010, Senut *et al.,* 2004, Veldman *et al*., 2007, Veldman *et al.,* 2010).

Udvadia *et al*., 2008 identified a 3.6 kb *gap43* promoter fragment from pufferfish that was sufficient to direct endogenous zebrafish *gap43* gene expression during both development and regeneration. This compact promoter sequence promotes reporter gene expression that faithfully recapitulates endogenous *gap43* expression. Promoter analysis of the 3.6 kb pufferfish *gap43* sequence identified distinct promoter regions necessary for developmental expression as opposed to regenerative *gap43* expression. Specifically, a 708 bp proximal promoter region was sufficient to drive expression of *gap43* during nervous system development. However, the same 708 bp promoter fragment was insufficient to drive expression of *gap43* during optic nerve regeneration. A more distal 2.9 kb promoter fragment was required for driving regenerative *gap43* expression, which will be referred to henceforth as regeneration-specific fugu *gap43* promoter regions (Kusik *et al.,* 2010). Within these broader regeneration specific promoter regions, shorter DNA sequence elements were identified that

showed a high level of conservation among distantly related teleost species. These teleost conserved sequence elements were absent in the *gap43* promoter regions from chicken, mouse, rat and humans (Kusik *et al*., 2010). The promoter analysis studies on fugu *gap43* transgenic fish outline two important findings (1) *gap43* promoter regions required to drive developmental expression are distinct from regions required to drive regenerative *gap43* expression (2) *cis*-acting DNA elements within regeneration-specific *gap43* promoter regions show a high degree of conservation among teleosts, which are capable of functional CNS regeneration, but are absent in higher vertebrates that fail to recover from CNS injury. These *cis*- acting elements are therefore potential targets of signaling pathways driving successful CNS regeneration and could explain species-specific differences in regeneration ability.

Candidate transcription factors predicted to drive regenerative gap43 expression

Kusik *et al*., 2010 identified regeneration-specific promoter regions within the pufferfish *gap43* promoter fragment. *In silico* analysis identified putative binding sites for several transcription factors within the regeneration specific promoter regions. We narrowed our focus to five candidate transcription factors likely to regulate *gap43* expression and axon outgrowth during optic nerve regeneration in fish. These transcription factors, henceforth referred as candidate transcription factors, are Ascl1a, Atf3, cJun, Stat3 and p53. While most of these transcription factors have been well studied in their ability to drive regeneration in the peripheral nervous system, a direct test to determine necessity for these factors during central nervous system regeneration is largely missing. In addition, very

few if any, downstream targets have been identified for these transcription factors during central nervous system regeneration. The next section summarizes the current consensus on: (1) activation of candidate transcription factors in response to injury, (2) functions of candidate transcription factors during nervous system regeneration, and (3) known downstream targets during nervous system regeneration.

cJun

Transcription factor cJun binds to AP-1 element in the form of heterodimers or homodimers. Common cJun binding partners include c-fos and members of the ATF/CREB family of transcription factors. cJun is found in basal levels in uninjured neurons and is strongly upregulated in response to several injury related signals such as growth factors, cytokines and stress factors (Herdegen *et al*., 1997). Retrogradely transported JNKs are responsible for the activation of cJun through phosphorylation of cJun N-terminal (Lindwall and Kanje, 2005) (Fig 1). Absence of cJun leads to impaired facial nerve regeneration in the PNS concomitant with a loss in expression of regeneration associated genes *cd44*, *galn* and *itga7* (Herdegen et al., 1997; Raivich et al., 2004; Lindwall and Kanje, 2005; Teng and Tang, 2006).

In the CNS, forced overexpression of cJun leads to increased CNS axon growth in cortical neuron slices (Lerch *et al*., 2014). This enhanced growth was however not accompanied by increases in *gap43* or *itga7*, both predicted targets of cJun based on observations in PNS regeneration (Lerch *et al*., 2014). There are

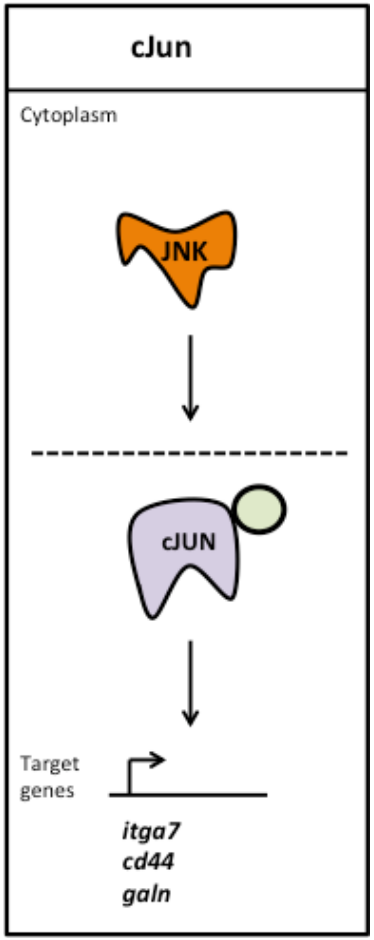
currently no known downstream targets of cJun that could explain effects observed on axon growth due to cJun over-expression in the CNS (Fig 2).

Atf3

Activating transcription factor-3 (Atf3) belongs to the ATF/CREB family of transcription factors and is capable of forming heterodimers with both members of leucine zipper proteins (CREB, cJun) and non-leucine zipper members (p53, STAT3) (Tedeschi *et al*., 2009). Atf3 expression is strongly upregulated in response to injury in neuronal populations capable of functional recovery such as peripheral branch of DRGs (Tsujino *et al*., 2000) and zebrafish retinal ganglion cells (Saul *et al*., 2010), but not in neurons incapable of functional regeneration (Tsujino *et al*., 2000). Similar to expression of cJun, extracellular injury signals such as JNK, p38 and ERK pathways are known to induce Atf3 expression in response to axonal injury (Hunt *et al*., 2010) (Fig 3). Activity of Atf3 is primarily regulated at the translational level and there is no evidence for transcriptional regulation of Atf3. However, the co-expression of cJun and Atf3 in the nervous system following stress signals and traumatic injury makes it tempting to speculate that cJun could be involved in transcriptional regulation of Atf3 during regeneration. However a requirement for cJun in regenerative induction of Atf3 expression is not previously demonstrated.

Evidence for Atf3 involvement in peripheral nerve regeneration comes from a study by Seijjfers *et al*., 2007 where Atf3 over-expression enhanced axonal sprouting in a facial nerve axotomy model. *sprr1*a and *hsp27* are the only known targets of Atf3 in the PNS (Seijjfers *et al*., 2007). Atf3 is upregulated by CNS

A

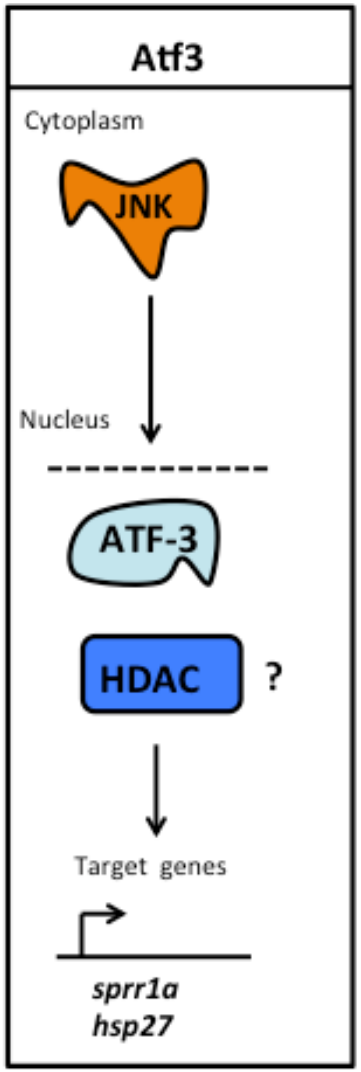


B

	Model of regeneration	Observed phenotypes	Target genes	References
Peripheral nervous system	Facial nerve axotomy – Mice	Strongly reduced target re-innervation Delayed functional recovery Decreased RAG expression and neuronal sprouting Strongly reduced glial activation and leukocyte recruitment Enhanced motor neuron survival Cellular atrophy	*itga7* *cd44* *galn*	Raivich *et al.*, 2004 Ruff *et al.*, 2012 Makwana *et al.*, 2010
	Sciatic nerve crush – Mice	Impaired axon regeneration		Saijilafu *et al.*, 2011
Central nervous system	Cortical slice cultures - Mice	Overexpression of cJUN promotes CNS neuron axon growth	?	Lerch *et al.*, 2014

Fig. 2. **Transcriptional pathways involving transcription factor c-Jun during regeneration** (A) Transcription factor c-Jun is activated through phosphorylation by JNK in response to injury. cJun activation results in regulation of genes *itga7*, *cd44* and *galn* during peripheral nerve regeneration (Adapted from Tedeschi *et al.*, 2012) (B) Table summarizing known effects and targets of transcription factor c-Jun in peripheral and central nervous system regeneration

A



B

	Model of regeneration	Observed phenotypes	Target genes	References
Peripheral nervous system	Sciatic nerve crush – Mice	Enhanced speed of regeneration in mice constitutively expressing Atf3 in DRGs	*sprr1a* *hsp27*	Seijffers *et al.*, 2007 Lewis *et al.*, 1999
Central nervous system	Peripheral nerve grafts in the thalamus of adult rats	Injured CNS neurons transiently upregulate ATF-3 expression	?	Campbell *et al.*, 2005
	Peripheral nerve grafts in the cervical spinal cord	Rubrospinal neurons growing into peripheral grafts upregulate ATF-3 expression		Shokouhi *et al.*, 2010
	Optic nerve regeneration-zebrafish	Injured RGCs strongly upregulate ATF-3 expression in response to optic nerve injury		Saul *et al.*, 2010

Fig. 3. Transcriptional pathways involving transcription factor Atf3 during regeneration (A) Transcription factor Atf3 is activated by retrograde injury signals in response to injury (Adapted from Tedeschi *et al.*, 2012). Atf3 activation results in regulation of genes *sprr1a* and *hsp27* during peripheral nerve regeneration (B) Table summarizing known effects and targets of transcription factor ATF-3 in peripheral and central nervous system regeneration

neurons growing into peripheral grafts, but a direct test of requirement for Atf3 during CNS regeneration has not been carried out.

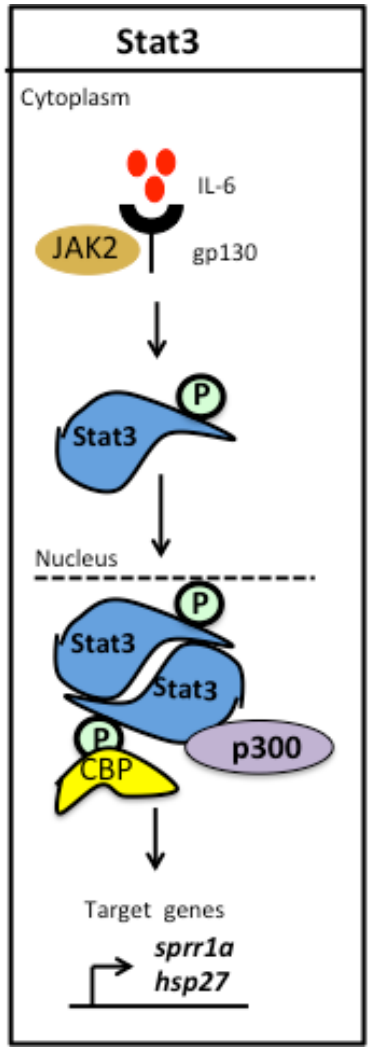
Stat3

Signal transducer and activator of transcription – 3 (Stat3) belongs to the STAT family of transcription factors. Stat3 is locally synthesized at the injury site and retrogradely transported as an injury signal that then signals the start of a gene program supportive of axon growth. Injury-induced cytokine IL6 binds to receptor gp130 that activates receptor tyrosine kinase JAKs leading to Stat33 activation through phosphorylation on residues 705 and 727 (Bromberg and Darnell 2000, Dziennis and Alkayed, 2008) (Fig 4). Stat3 knockout mice displayed strongly reduced target re-innervation and delayed functional recovery following saphenous nerve lesion. In addition, overexpression of Stat3 improves collateral and terminal sprouting in the central branch of DRG following transection in a phase-specific manner (Baryere *et al*., 2011). It is currently not known how effects of Stat3 on axon re-growth are mediated in the nervous system following injury. However, Stat3 null mice display reduced levels of *sprr1a* and *hsp27*, hinting that they are potential downstream targets (reviewed by Patodia and Raivich *et al*.,2012) (Fig 4).

p53

p53 belongs to the family of tumor suppressors commonly known for mediating both pro- and anti-apoptotic roles in the nervous system (Jacobs *et al.,* 2006). p53 is regulated at the post-translation level through modifications such as acetylation, phosphorylation and ubiquitination that influences localization and

A



B

	Model of regeneration	Observed phenotypes	Target genes	References
Peripheral nervous system	Saphenous nerve lesion-Mice	Strongly reduced target re-innervation Delayed functional recovery	*sprr1a* *hsp27*	Bareyre *et al.*, 2011
	Facial nerve axotomy-Mice	Decreased RAG expression and neuronal sprouting Strongly reduced glial activation and leukocyte recruitment Enhanced motor neuron survival Cellular atrophy		Patodia *et al.*, 2011
Central nervous system	DRG central branch lesion	Overexpression of Stat3 improves collateral and terminal sprouting through induction of axon growth. Stat3 not required for elongation	?	Bareyre *et al.*, 2011

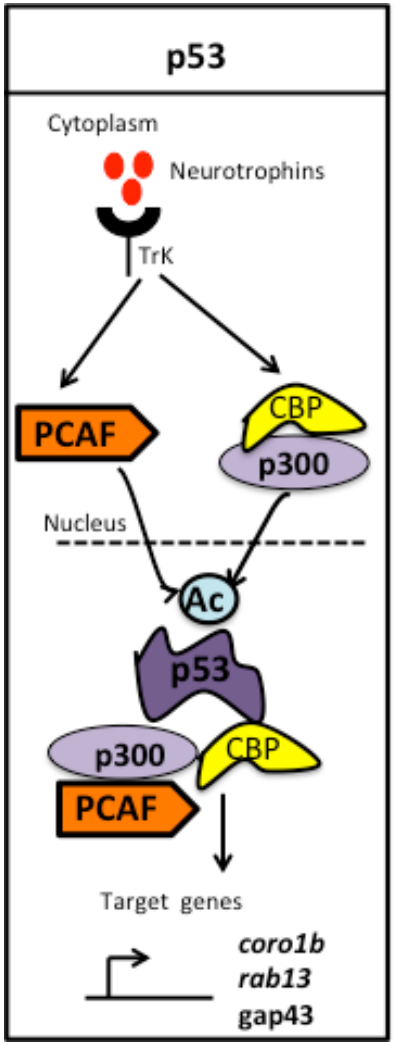
Fig. 4. Transcriptional pathways involving transcription factor Stat3 during regeneration. (A) Transcription factor Stat3 is activated by receptor ligand binding that leads to activation of associated JAK2. JAK2 activation results in phosphorylation of Stat3 leading to activation and translocation into nucleus. Phosphorylated Stat3 dimerizes and along with co-activators leads to regulation of genes *sprr1a* and *hsp27* during peripheral nerve regeneration (Adpated from Tedeschi *et al.*, 2012) (B) Table summarizing known effects and targets of transcription factor Stat3 in peripheral and central nervous system regeneration

function (Tedeschi *et al*., 2009) (Fig 5). p53 was found to be an essential component of peripheral nerve regeneration by modulating expression of regeneration associated genes *coro1b, rab12 and gap43* (DiGiovanni *et al*., 2006, Tedeschi *et al*., 2009) (Fig 5). Requirement for p53 during CNS regeneration has not been tested yet. (Fig 5)

Ascl1

Achaete-scute complex-like 1a (Ascl1a) belongs to the basic helix loop helix family of transcription factors. Ascl1a is one of the earliest genes to be induced in response to optic nerve injury , with expression detected as early as 4 hours post injury (Fausett *et al*., 2008). Ascl1a is expressed in neural progenitors in chick, mouse and zebrafish (Fisher and Reh 2001, Yun *et al.,* 2002, Yurco and Cameron 2007), suggesting a role in cell fate determination and specification.

Ascl1a was found to activate expression of regeneration associated gene *tuba1a* via an E-box element during optic nerve regeneration in zebrafish. Ascl1a knockdown also affected axon re-growth in dissociated retinal cultures (Fausett *et al*., 2008). Ascl1a influences the ability of Mueller glia to de-differentiate to a progenitor state to replace dead neurons in zebrafish (Fausett *et al*., 2008). However whether Ascl1a is required for axon re-growth of surviving neurons during CNS regeneration remains unknown.



	Model of regeneration	Observed phenotypes	Target genes	References
Peripheral nervous system	Facial nerve axotomy – Mice	Decreased neurite outgrowth Reduced target innervation	*coro1b* *rab13* *gap43*	Di Giovanni *et al.*,2006 Qin *et al.,* 2009 Tedeschi *et al.*, 2009
Central nervous system	Not tested	Not tested	?	-

Fig. 5. Transcriptional pathways involving transcription factor p53 during regeneration. (A) Transcription factor p53 is activated by receptor ligand binding leading to post translational modifications by p300/CBP and PCAF. Acetylated p53 forms a transcriptional module along with p300/CBP that occupies promoters of regeneration associated genes *coro1b*, *rab13* and *gap43* and regulates expression during regeneration (Adapted from Tedeschi *et al.*, 2012) (B) Table summarizing known effects and targets of transcription factor p53 in peripheral and central nervous system regeneration

Thesis statement

In contrast to mammals, zebrafish respond to CNS injury by successfully upregulating essential regeneration-associated genes and mounting a functional regenerative response. This makes them ideal models to uncover gene regulatory pathways underlying successful CNS regeneration. To gain insight into mechanisms underlying successful CNS regeneration, we have studied the regulation of the *gap43* gene as a model regeneration-associated gene, to identify specific transcriptional complexes required for *in vivo* axon regeneration.

Although *gap43* over-expression drives modest outgrowth in neurons incapable of regeneration, experiments described in this thesis demonstrate evidence for the first time that re-expression of Gap43 is required for successful regeneration in neurons capable of functional recovery. We have identified a conserved role for transcription factors Ascl1a, Atf3, cJUN and Stat3 in driving regenerative *gap43* expression and axon outgrowth during CNS regeneration in fish. Furthermore, we have found that three of these transcription factors, Ascl1a, Atf3, cJUN, seem to function in a synergistic, rather than additive manner, suggesting that the absence of any one is sufficient to prevent regeneration. These findings extend our basic understanding of neuron-intrinsic mechanisms underlying successful CNS regeneration and reveal potential therapeutic targets for manipulation to improve CNS regeneration in mammals.

Chapter II

Materials and Methods

Zebrafish husbandry and reporter lines

Zebrafish husbandry and all experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Wisconsin-Milwaukee and were performed in accordance with animal welfare standards established by the US National Institutes of Health guide for the care and use of laboratory animals.

Zebrafish colonies were maintained as previously described (Kusik et al., 2010). Adult zebrafish were maintained at 28°C with a 14-hour light/ 10-hour dark cycle, and fed twice daily with Artemia as well as Zeigler Adult Zebrafish Complete Diet (VWR, West Chester, PA). Two strains of zebrafish were used in these experiments: 1) Ekkwill, a wild type strain, used in the RGC axon regeneration assays, and 2) Tg (*Tru.gap43:egfp*) mil1, a.k.a. *fgap43:egfp*, a fugu *gap43* transgenic reporter strain constructed on the Ekkwill background (Udvadia et al., 2008) used in the *gap43* gene expression studies.

Zebrafish optic nerve injury and gene knockdown

Targeted knockdown of mRNAs in regenerating retina ganglion cells was accomplished by introducing gene-specific MOs (MOs) delivered by retrograde axonal transport from the site of optic nerve injury. Adult zebrafish were anesthetized with 0.03% aminobenzoic acid ethylmethylester (Argent Chemical

Labs, Redmond, WA), and immobilized in a foam mold. The left optic nerves were fully transected one mm from the retina. The right eyes were left intact to serve as unoperated controls. Gene knockdown was accomplished by placing a 1mm sized piece of gel foam soaked with MOs (Gene tools LLC, Philomath, OR) at the site of optic nerve transection. Sequences used for MO synthesis are summarized in Table 2. All MOs were tagged with the red fluorescent tracer, lissamine. This allowed for identification of neurons that received MOs through retrograde transport.

Table 2. List of morpholinos used in the study

Gene target	Sequences used for synthesis	Target site, References
Ctrl	5' CCTCTTACCTCAGTTACAATTTATA 3'	Veldman *et al*., 2007
gap43	5' TCTTCTGATGCAGCACAGCATAGTC 3'	Translation start site
ßascl1a	5′-AAGGAGTGAGTCAAAGCACTAAAGT-3′	Translation start site (Cau and Wilson, 2003)
atf3	5' AGTAAATGAGTGGGTCTTACCTCTC 3'	Splice donor site between exons 3 and 4
jun	5' TCCATCTTGGTAGACATAGAAGGCA 3'	Translation start site
stat3	5' CATTTCCAATGCAGTCATACCTCCA 3'	Exon5/Intron 5 boundary
p53	5' GCGCCATTGCTTTGCAAGAATTG 3'	Translation start site

Reporter gene assays and MO verification in regenerating retinas

For the gene expression assays, 6-9 month old *fgap43:egfp* were subjected to optic nerve injury as described above. Animals were sacrificed four-days post-injury, and retinas were removed, fixed, and prepared for frozen sectioning as previously described (Kusik et al., 2010). Transverse sections (10 µm) were

collected on glass slides and cover slipped in Vectashield Hard Set mounting medium (Vector Labs, Burlingame, CA) with DAPI to stain the nuclei. Fluorescent images were obtained using a Zeiss Apotome microscope (Carl Zeiss AG, Oberkochen, Germany). Cells expressing *gap43* were identified by the green fluorescence from the fugu *gap43:egfp* transgene (Udvadia 2008).

MO-mediated mRNA knockdown of targeted transcription factors was verified in regenerating retinas at four days post-injury using immunofluorescence staining. MO treatments and preparation of retinas for cryosectioning were performed as described above. Slides were rehydrated in PBST (0.1% Tween 20, 2XPBS) for 3 min and incubated in blocking buffer (10%NCS/PBST) for 1 hour at room temperature prior to incubating overnight at 4°C in primary antibody diluted in blocking buffer. The primary antibodies and corresponding concentrations used for these studies are listed in Table 3. Following overnight incubation with primary antibody, slides were washed with PBST for 10 min at room temperature 3 times and then incubated with secondary antibodies (Alexa Fluor 488, Invitrogen, Grand Island, NY) diluted 1:500 in PBST for 2 hours at room temperature. Following incubation with secondary antibodies, slides were washed again with PBST before mounting with VECTASHIELD (Vector labs, Burlingame, CA) to stain the nuclei. Fluorescent images were obtained using a Zeiss Apotome microscope.

Table 3. List of primary antibodies used in the study		
Protein	**Vendor information**	**Primary antibody concentration**
Atf3	C-19, Santa Cruz Biotechnology, Santa Cruz, CA	1:100
cJun	31419, Abcam, Cambridge, MA	1:100
Stat3	SC-H190, Santa Cruz Biotechnology, Santa Cruz, CA	1:100
p53	GTX128135, GeneTex, Irvine, CA	1:100
HuC/HuD	A21721, Molecular probes, Grand Island, NY	1:500
Caspase 3	AF835, R&D systems Inc., R&D R&D systems Inc., Minneapolis, MN	1:1000

Quantification of gene expression

We used quantitative PCR (QPCR) to quantify the effect of knocking down regeneration-associated transcription factors on expression of *fgap43:egfp* transgene and endogenous zebrafish *gap43* gene. QPCR was performed on an ABI 7500 Fast Real time PCR system (Applied Biosystems, Carlsbad, CA) with SYBR green fluorescent label (Quanta Biosciences, Gaithersburg, MD). Total RNA was isolated from adult retina of *fgap43:egfp* zebrafish using Trizol reagent (Invitrogen, Grand Island, NY). The cDNA was synthesized from 550 ng total RNA with Oligo dT priming using qSCRIPT reverse transcriptase (Quanta Biosciences, Gaithersburg, MD). The QPCR analysis was performed to determine the relative levels of *gap43* and *gfp* mRNA in each sample, using *ef1*α as an internal control.

We used qPCR for the transcription factor inter-regulation analyses, to quantify expression of *ascl1a, atf3* and *jun*, following knockdown of either of the other two transcription factors. Samples were prepared as described above.

Primer sequences used for each gene are summarized in Table 4. A dissociation step was performed at the end of the amplification phase to confirm a single, specific melting temperature for each primer set. Cycle threshold values (Ct) were normalized to *ef1*α as an internal reference.

Relative gene expression was quantified using the 2^-(ΔΔCt) method (Livak and Schmittgen, 2001), ΔCt1 = Normalized Ct (operated Lt eye) and ΔCt2 = Normalized Ct (unoperated right eye). A similar analysis was performed on retinas treated with negative control MOs for comparison. Normalized gene expression data from 3-4 biological replicates were averaged and analyzed as fold change.

Table 3. List of primers used in the study

Gene	Forward primer	Reverse primer
gap43	5'- CCAAAGAGGAAGTGAAGGAG-3'	5'-CAGCAGCGTCTGGTTTGTC-3'
gfp	5'-AACGAGAAGCGCGATCAC-3'	5'CCATAGGTTGGAATCTTAGAG-3'
efl1α	5'- GTACTTCTCAGGCTGACTGTG-3'	5'-CGCTGACTTCTTGGTGAT-3'
atf3	5' TCACGCTGGACGACTTCACAAACT 3'	5'TCTCAGTGTTCATGCAGGCTCTGT 3'
ascl1a	5' GACGAGCATGACGCCGTAAG 3'	5'AAGTTTCCTTTTACGAACGCTCAA 3'
jun	5' ACATCGACCAGGTTGTGCAT 3'	5' CGCGTCCCTGTTTTACTCCT 3'

Optic nerve regeneration assays

The RGC axon regeneration assays were performed as previously described by Zou *et al.,* 2013. 9 month – one year old wild type zebrafish were anesthetized and their left optic nerves were partially transected one mm from the retina. Then a piece of gel foam soaked with corresponding MOs was placed at the site of transection. After four days, regenerating axons were traced from the right optic tectum using a fluorescent retrograde tracer (DiI-N22880, Invitrogen, Grand Island, NY). Nine days after retrograde tracing, retinas were removed and prepared for frozen sectioning and fluorescence microscopy as described above. Images were collected from five sections per retina, which included sections from the center of the retina containing the optic nerve and two lateral sections on each side. RGCs that were injured and received the MO from the optic nerve transection were identified by the red fluorescence from lissamine. RGCs that regenerated their axons to the contralateral optic tectum were identified by green fluorescence from the tracer. The percentage of axon regeneration was calculated by counting the number of double fluorescent cells (yellow), divided by the total number of cells taking up the MO (red) and multiplied by 100.

Statistical analysis

All statistical analysis was done using Graph Pad Prism 6 software. For the qPCR assays and the axon regeneration assays, values from control MO treated retinas were pooled together and outlier analysis was performed (Mean ± 2STDEV) to ensure that data values fall within the normal statistical range before being used for further analysis. Individual transcription knockdown values were

then compared to the pooled controls and analyzed by one-way analysis of variance (ANOVA) followed by the Tukey post-hoc method for pair-wise multiple comparisons.

CHAPTER III

Identification of transcription factors regulating *gap43* gene expression and axon outgrowth during optic nerve regeneration in zebrafish

Central nervous system (CNS) injury results in the disruption of neuronal networks and permanent disability in mammals, however, the ability to regenerate damaged CNS nerves and achieve functional regeneration occurs naturally in fish. While it is true that the extracellular environment of the mammalian CNS contains growth inhibitory factors that prevent regeneration, simply removing the inhibitory factors is not sufficient to promote functional regeneration (reviewed in Ferguson and Son, 2011). Thus the focus in regenerative research has returned to the investigation of neuron-specific factors that promote the ability of mammalian CNS neurons to regenerate.

The re-expression of neuronal growth-associated genes after CNS injury occurs naturally in fish, yet our knowledge of proteins and regulatory pathways that are actually *necessary* for CNS regeneration in fish remains limited. To identify molecular mechanisms underlying successful CNS regeneration in fish, we have focused on the regeneration-associated regulation of the growth-associated protein 43 (Gap43), a prototypical axon growth protein encoded by the *gap43* gene. The importance of *gap43* gene expression in facilitating regeneration is

evident from studies in which ectopic expression of Gap43 in combination with other neuronal growth associated proteins (nGAPs) induces axon regrowth in neuronal populations previously incapable of regeneration (Bomze et al., 2001; Zhang et al., 2005).

In zebrafish, levels of *gap43* mRNA and protein are rapidly increased in response to injury and remain elevated through the entire phase of regenerative growth (Bormann *et al.*,1998; Kaneda *et al.*,2008). However, the requirement of *gap43* expression for successful CNS regeneration in fish has not been established. Previous studies from our lab identified specific regions within the pufferfish (fugu) *gap43* promoter that are indispensable for regenerative *gap43* expression and highly conserved across divergent teleost species (Kusik et al., 2010). These regeneration specific promoter regions harbor putative binding sites for several transcription factors. While these transcription factors were previously implicated in axon growth and regeneration, none of them had been tested for their requirement in successful CNS axon regeneration. We have used an optic nerve transection injury in adult zebrafish to model successful vertebrate CNS regeneration in order to address the following questions:

1) Is *gap43* induction following optic nerve injury necessary for successful regeneration?

2) Are the candidate transcription factors with putative binding sites in the fugu *gap43* regeneration-specific promoter regions necessary for *gap43* expression in following optic nerve injury?

3) Are the candidate transcription factors with putative binding sites in the fugu *gap43* regeneration-specific promoter regions necessary for successful regeneration following optic nerve injury?

We hypothesized that both Gap43 and the transcription factors that regulate its expression in regenerating CNS neurons would be essential for regenerative axon growth leading to successful CNS target re-innervation after optic nerve transection. Using *in vivo* reporter assays and *in vivo* regeneration assays, we have determined that Gap43 expression is required for the successful regrowth of retinal ganglion cell axons and re-innervation of the optic tectum following optic nerve transection in zebrafish. Furthermore, we have identified specific transcriptional complexes that are required for both re-initiating *gap43* gene expression and promoting regenerative axon outgrowth in response to optic nerve injury. The data contained in this chapter are part of two separate manuscripts in preparation (Williams et al., *in preparation*; Venkatesh and Udvadia, *in preparation*).

Verification of gene knockdown approach for specificity and efficacy

We used morpholino-mediated gene knockdown of Gap43 and candidate transcription factors regulating *gap43* gene expression in regenerating CNS neurons to determine their roles in successful CNS regeneration. Morpholinos (MOs) are synthetic, stable oligonucleotides designed to hybridize with a target mRNA and prevent protein synthesis by blocking translation or mRNA processing. Ascl1a, cJun and p53 MOs were targeted to the translation start site.

atf3 MO targeted the splice donor site between exons 3 and 4 and *stat3* MO targeted the exon5/intron5 boundary. MOs were delivered by retrograde transport from the site of injury. All MOs were conjugated with a fluorescent lissamine label to allow verification of MO delivery to the RGCs. In the following series of experiments we established that the MOs resulted in specific knockdown of the targeted genes and did not have non-specific toxic effects on RGCs.

The specificity and efficacy of MOs in knocking down expression of their target genes was validated using either transgenic zebrafish expressing reporter genes targeted by the MOs, or immunofluorescence staining. In each case targeted knockdown by MOs was compared with control MO that targets a human sequence not present in zebrafish. A transgenic zebrafish line expressing a *gap43-GFP* fusion protein (Udvadia, 2008) was used to verify the efficacy and specificity of the *gap43* MO. The *gap43-GFP* transgene used in the study contains the promoter/enhancer sequences upstream of the fugu *gap43* gene in addition to exon 1 sequences that include the 5' untranslated region (UTR) as well as sequences encoding the first 10 amino acids of the protein fused to the coding sequence for GFP. The resulting transgene encodes a GFP fusion protein that is targeted by the *gap43* MO. Retinas treated with control or *gap43* MO were sectioned and imaged for MO uptake (red fluorescence), and expression of the *gap43-GFP* transgene (green fluorescence) in the RGCs. Both the control and *gap43* MOs were effectively taken up by the RGCs, however; only *gap43* MO was able to knockdown *gap43-GFP* expression (Fig. 6A, B). Similarly, the

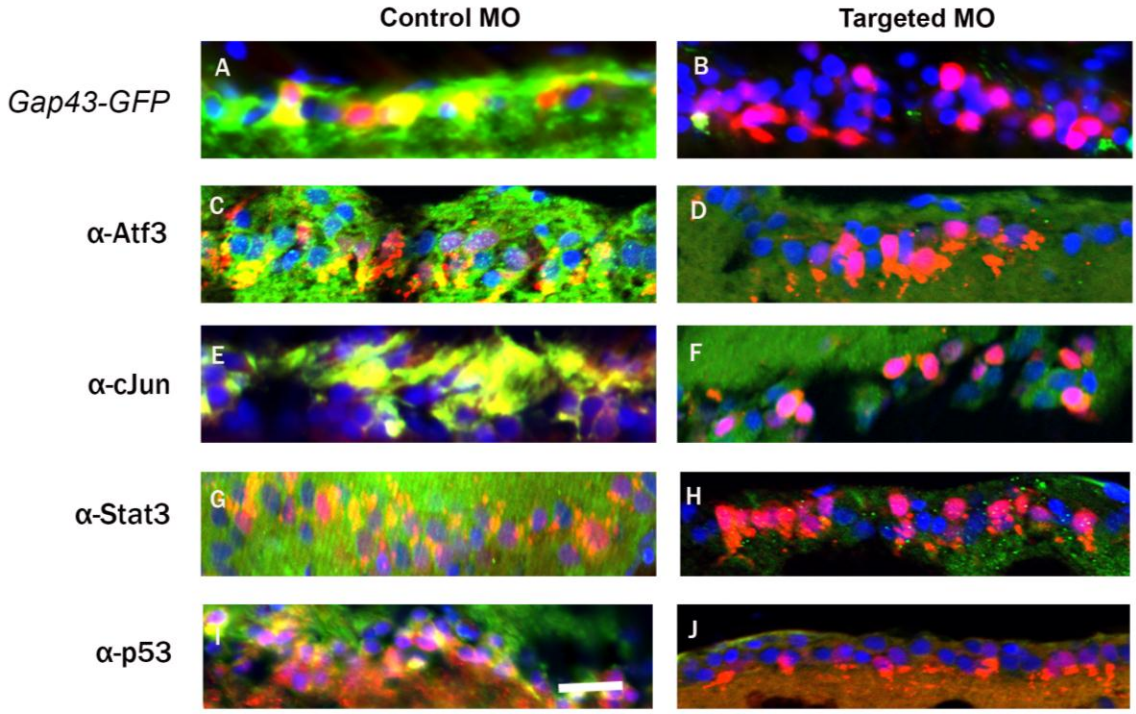


Fig. 6. Morpholinos targeting Gap43 and candidate transcription factors are effective and specific. Representative images of the RGC layer from transverse sections through regenerating adult retinas treated with either control MO (A, C, E, G, I) or MOs targeting candidate proteins (B, D, F, H, J) and preserved four days after optic nerve transection. The control MO does not affect target protein expression (A, C, E, G, I), while targeted knockdowns results in a substantial decrease in target protein levels (B, D, F, H, J). Blue, DAPI; Red, lissamine-labeled MOs; Green, GFP (A, B) or Alexa 488 secondary antibody (C-J). Scale bar = 20 μm.

efficacy of the previously described *ascl1a* MO used in these studies was verified by knockdown of an *ascl1a-GFP* fusion protein expressed in zebrafish embryos (Cau and Wilson, 2003). Gene knockdown by MOs targeting *atf3, cjun, stat3, and p53* was validated using immunofluorescence staining with antibodies specific to each of the transcription factors. In each case, we observed antibody staining in retinas receiving the control MO, indicating that the MO delivery did not interfere with injury-induced upregulation of the transcription factors (Fig. 6C, E, G, I). As expected, we observed little to no antibody staining after gene specific knockdown (Fig. 6D, F, H, J). Together these data validate the effective and specific knockdown of injury-induced protein expression by MOs targeting transcripts for *gap43* and candidate transcription factors.

Unlike in mammals, optic nerve injury in fish does not normally result in an increase in RGC cell death. In order to rule out the possibility of MO toxicity to the RGCs, we verified that MO delivery did not cause RGC cell death. We observed no activated caspase-3 staining in RGCs indicating that MO uptake did not lead to cell death (Fig. 7). Thus, we conclude that retrograde delivery of MOs does not have a non-specific effect on survival of regenerating RGCs.

Since most of the genes targeted by the MOs are also important in neuronal differentiation, we wanted to verify that MO-mediated knockdown of these genes did not interfere with the maintenance of the neuronal phenotype. We used immunostaining to verify that MO uptake by RGCs did not affect expression of the HuC/HuD antigen, a common pan-neuronal marker. Based on the normal

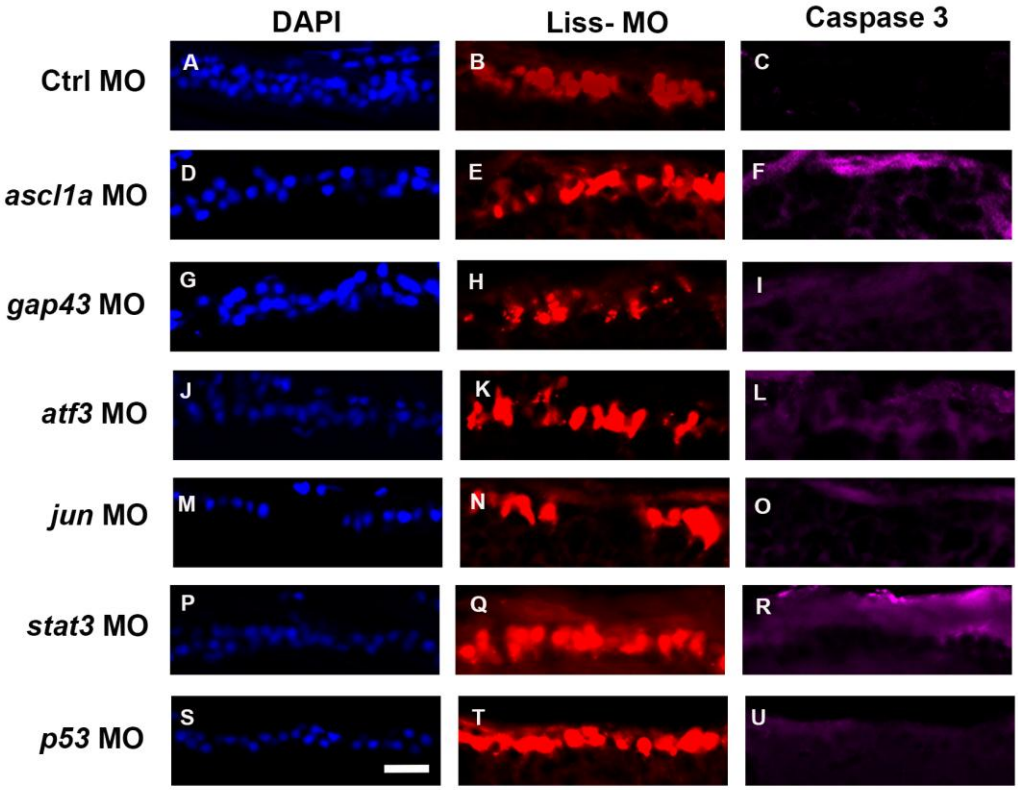


Fig. 7. Uptake of morpholinos (MOs) does not result in increased RGC cell death. Representative images of the RGC layer from transverse sections through regenerating adult retinas treated with control or targeted lissamine-labeled MOs (**B, E, H, K, N, Q, T**) and preserved four days after optic nerve transection. The effect of MO-mediated gene knockdown on RGC cell death was assessed by immunostaining with zebrafish caspase-3 antibody (**C, F, I, L, O, R, U**). Nuclei of all cells were stained with DAPI (**A, D, G, J, M, P, S**). Absence of caspase-3 co-localization in RGCs confirms that MO uptake does not result in increased RGC cell death. Scale bar = 20 μm

expression of the HuC/D in RGCs, we concluded that MO uptake did not affect neuronal identity (Fig 8).

Gap43 expression is required for optic nerve regeneration *in vivo*

Gap43 is a prototypical axon growth-associated protein, which is highly expressed in developing and regenerating axonal growth cones. Gap43 expression is transcriptionally downregulated in most mature neurons. Previous studies have demonstrated that ectopic expression of Gap43 along with one other growth-associated protein (either Cap23 or L1) in the adult mammalian CNS is sufficient to stimulate regrowth of axons that normally lack regenerative capacity (Bomze *et al.*, 2001, Zhang *et al.*, 2005). However the requirement for Gap43 in CNS neurons that are capable of fully regenerating severed axons has not been investigated.

To test the hypothesis that *gap43* re-expression is necessary for CNS regeneration *in vivo*, we assessed the ability of retinal ganglion cell (RGC) axons to regrow to the optic tectum in adult zebrafish following MO-mediated knockdown of *gap43* expression. MOs were delivered via retrograde transport at the time of optic nerve injury as described above (Fig. 9A). A fluoresceinated dextran tracer was deposited on the contralateral tectum four days after the original optic nerve injury to label only the RGCs that had successfully regenerated axons and reinnervated the target tissue (Fig. 9B). Thirteen days post-injury, retinas were dissected and prepared for cryosectioning and fluorescence imaging (Fig. 9C). Injured RGCs that had taken up the MO were identified by virtue of the red fluorescence from the lissamine tag (Fig. 10B, F, J). RGCs with axons that had regenerated to the optic tectum were identified by their uptake of fluoresceinated dextrans, which appears yellow in the merged image (Fig 10C, G, K). We quantified the percent of RGCs that successfully regenerated by counting the

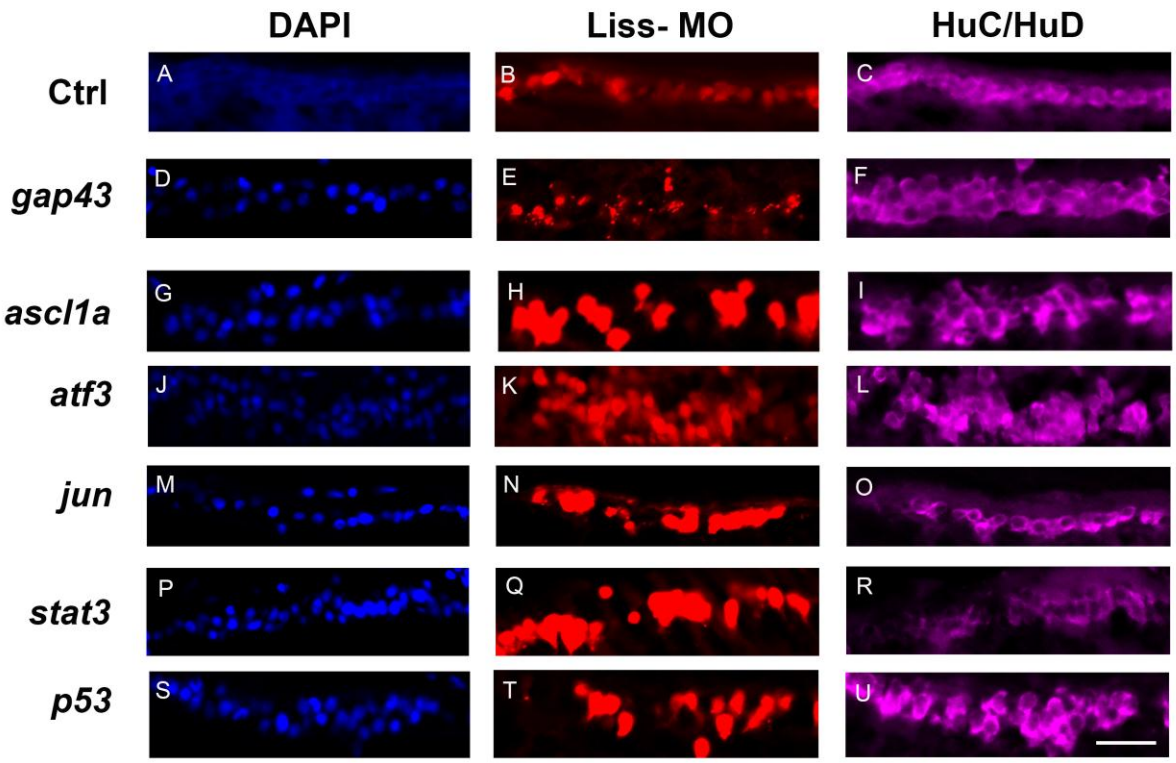


Fig. 8. Knockdown of regeneration-associated transcription factors does not alter neuronal identity. Representative images of the RGC layer from transverse sections through regenerating adult retinas treated with control or targeted lissamine-labeled MOs (**B, E, H, K, N, Q, T**) and preserved four days after optic nerve transection. The effect of MO-mediated gene knockdown on neuronal identity was assessed by immunostaining with zebrafish HuC/HuD antibody (**C, F, I, L, O, R, U**). Nuclei of all cells were stained with DAPI (**A, D, G, J, M, P, S**). Expression of the HuC/HuD antigen was unaffected by MO treatment, confirming normal neuronal identity of RGCs after transcription factor knockdown. Scale bar = 20μm

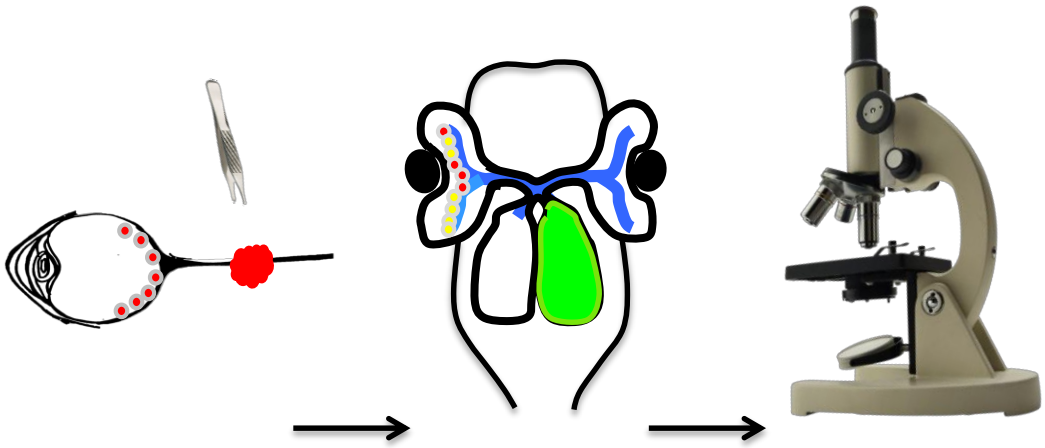


Fig. 9. Timeline for retinotectal regeneration assay. Lissamine-labeled morpholinos were delivered to the RGC cell bodies through retrograde transport at the time of optic nerve transection. Four days post-injury, fluoresceinated dextran was placed on the contralateral tectum to back-label regenerating RGC axons. Retinas were dissected 13 days post-injury, prepared for cryosectioning, and imaged using wide-field fluorescence microscopy.

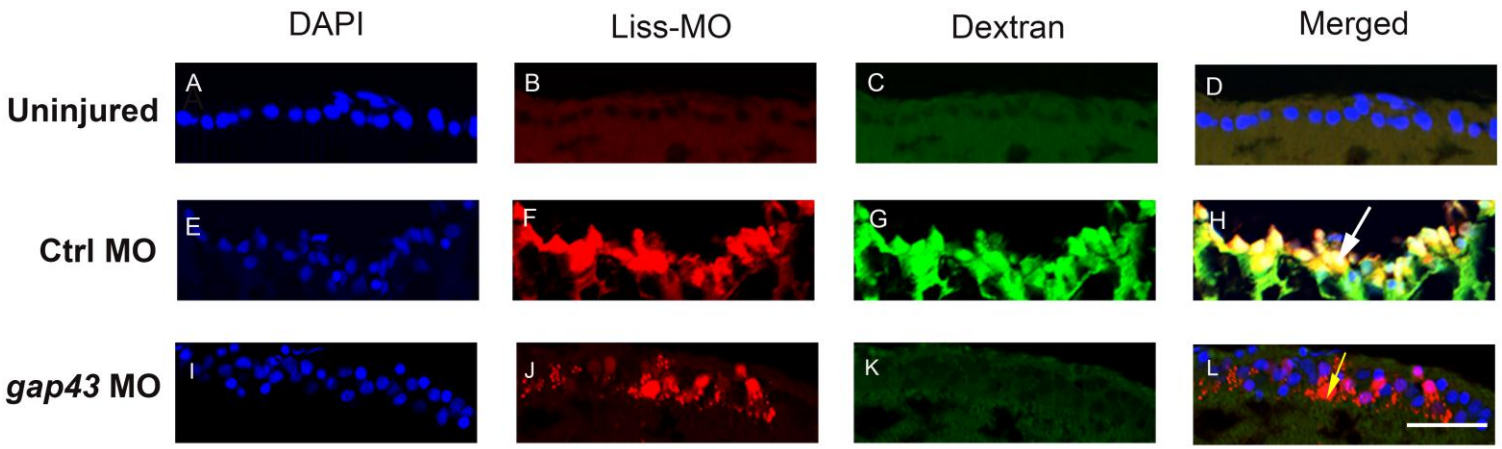


Fig. 10. *Gap43* knockdown disrupts retinotectal regeneration. Representative images of the RGC layer from transverse sections through uninjured retina (A-D), regenerating adult retinas treated with either control MO (E-H) or *gap43* MO (I-L). Lissamine-labeled morpholinos (red) were delivered to RGCs through retrograde axonal transport after proximal optic nerve transection (F, J). RGC axons regenerating to the tectum were retrogradely labeled with fluoresceinated dextran (green) four days post injury (C, G, K). Retinas were harvested 9 days after tectum labeling, cryosectioned and coverslipped with DAPI mounting medium to stain the nuclei (blue). RGCs treated with control MO displayed robust re-growth as evident by the co-localization of MOs and tracer (H, white arrow). In contrast, few RGCs regenerated in retinas treated with *Gap43* MO, as evident by lack of co-localization with tracer (L, yellow arrow). Scale bar = 20 μM

number of regenerated RGCs (fluorescein-dextran uptake, green/yellow) dividing by the number of injured neurons (lissamine-MO, red). We observed that 2-weeks post optic nerve transection, 50-60% of the injured RGCs treated with the control MO had regenerated to the contralateral tectum (Fig. 11). In contrast, MO-mediated downregulation of *gap43* resulted in a sharp decrease in number of RGCs re-growing to the tectum (Fig. 11), with only ~3% of injured neurons showing complete axon regeneration. These results establish that upregulation of the Gap43 protein in response to injury is essential for the successful re-establishment of retinotectal projections following optic nerve transection.

Transcription factors are differentially required for regenerative *gap43* reporter expression

Given the importance of injury-induced *gap43* expression in retinotectal regeneration, we next sought to identify the transcription factors responsible for regeneration-associated induction of the *gap43* gene. We previously demonstrated that a 3.6 kb *gap43* promoter fragment from pufferfish is sufficient to promote reporter gene expression during zebrafish development and after optic nerve injury in a manner that recapitulates endogenous zebrafish *gap43* expression (Udvadia *et al.*, 2008). Furthermore, we found that the regeneration-specific *gap43* promoter regions contained putative binding sites for transcription factors commonly implicated in axon growth (Fig. 12). Our studies focused on five transcription factors, Ascl1a, Atf3, cJun, Stat3, and p53, which had been shown to be important for axon growth in developing or cultured neurons, or during peripheral nervous system regeneration (Table 1).

We tested the ability of the candidate transcription factors to induce regenerative *gap43* expression by assaying the effects of transcription factor knockdown on *gap43* reporter gene activity in the injured adult zebrafish retina. As expected, the *gap43-GFP* reporter

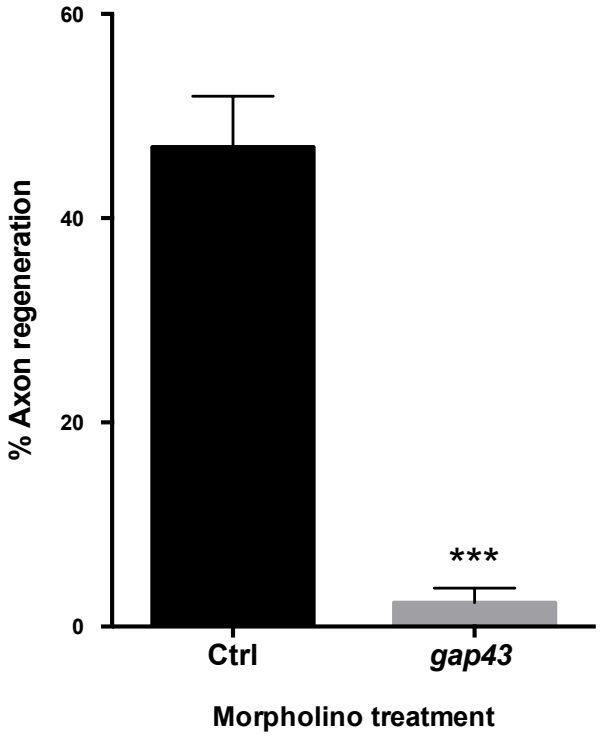


Fig. 11. Knockdown of *gap43* results in an ~20-fold decrease in RGC axon regeneration. The percentage of RGCs that regenerated axons to the optic tectum after *gap43* knockdown was calculated. In control MO treated RGCs, ~50% of the injured axons regenerated back to the tectum 2 weeks post injury. *gap43* MO treated RGCs display an ~20-fold decrease in axon regeneration, with 2.3% regeneration. n=4 error bars = SEM Statistical significance was assessed using t test ($p<0.001$).

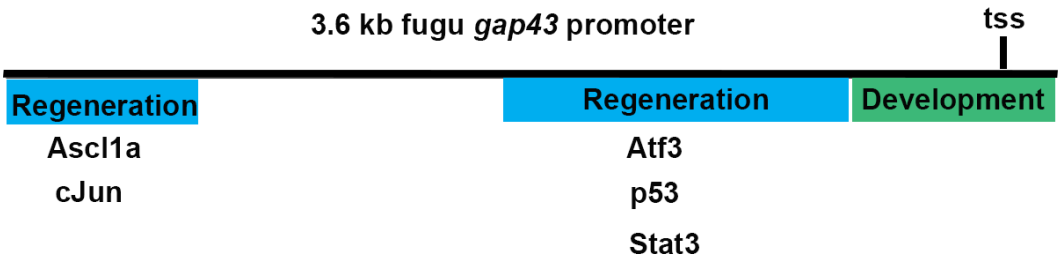


Fig. 12. Putative binding sites for regeneration-associated transcription factors in the fugu *gap43* gene promoter. Regions associated with developmental and regenerative *gap43* expression are color-coded. Putative transcription factor binding sites are indicated within regeneration specific promoter regions. tss- Transcription start site (*Adapted from Kusik et al., 2010*)

gene expression was strongly induced four days post-injury in retinas treated with the control MO (Fig. 13B), while RGCs treated with the *gap43* MO displayed a complete reduction in reporter gene expression (Fig. 13E). MO-mediated knockdown of the various transcription factors had varying effects on reporter gene expression. MOs targeting *ascl1a*, *atf3*, or *jun* all significantly prevented injury-induced *gap43* reporter gene expression (Fig. 13H, K, N). This result suggests that *ascl1a, atf3* and *jun* are acting in a synergistic manner rather than in an additive manner with regard to *gap43* activation. *Stat3* MO also significantly impacted injury-induced *gap43* reporter gene expression (Fig. 13Q), although not to the same extent as observed with *ascl1a, atf3* and *jun* knockdown. Surprisingly, knockdown of *p53* has no significant effect on injury-induced *gap43* reporter expression (Fig. 13T). We conclude that there is a differential requirement for the candidate transcription factors in driving regenerative *gap43* promoter expression.

We next quantified the relative contributions of the various candidate transcription factors to the upregulation of *gap43* reporter gene expression in regenerating RGCs. We isolated RNA from regenerating retinas treated with transcription factor MOs at 4 days post-injury and used quantitative PCR (QPCR) to determine the effects of transcription factor knockdown on injury-induced *gap43* reporter gene expression. Our QPCR results corroborated what we observed qualitatively in the fluorescence imaging experiments described above. We observed a 50-fold increase in *gap43* reporter expression in regenerating retinas compared to the uninjured contralateral control retinas (Fig. 14). In contrast, in retinas that received the *ascl1a* MO we observed virtually no reporter gene activity, while retinas receiving *atf3* or *jun* MOs displayed an approximately four to six-fold reduction in *gap43* reporter gene expression compared to controls (Fig. 14). Consistent with the visualization of the *in vivo* reporter assays, we found that although

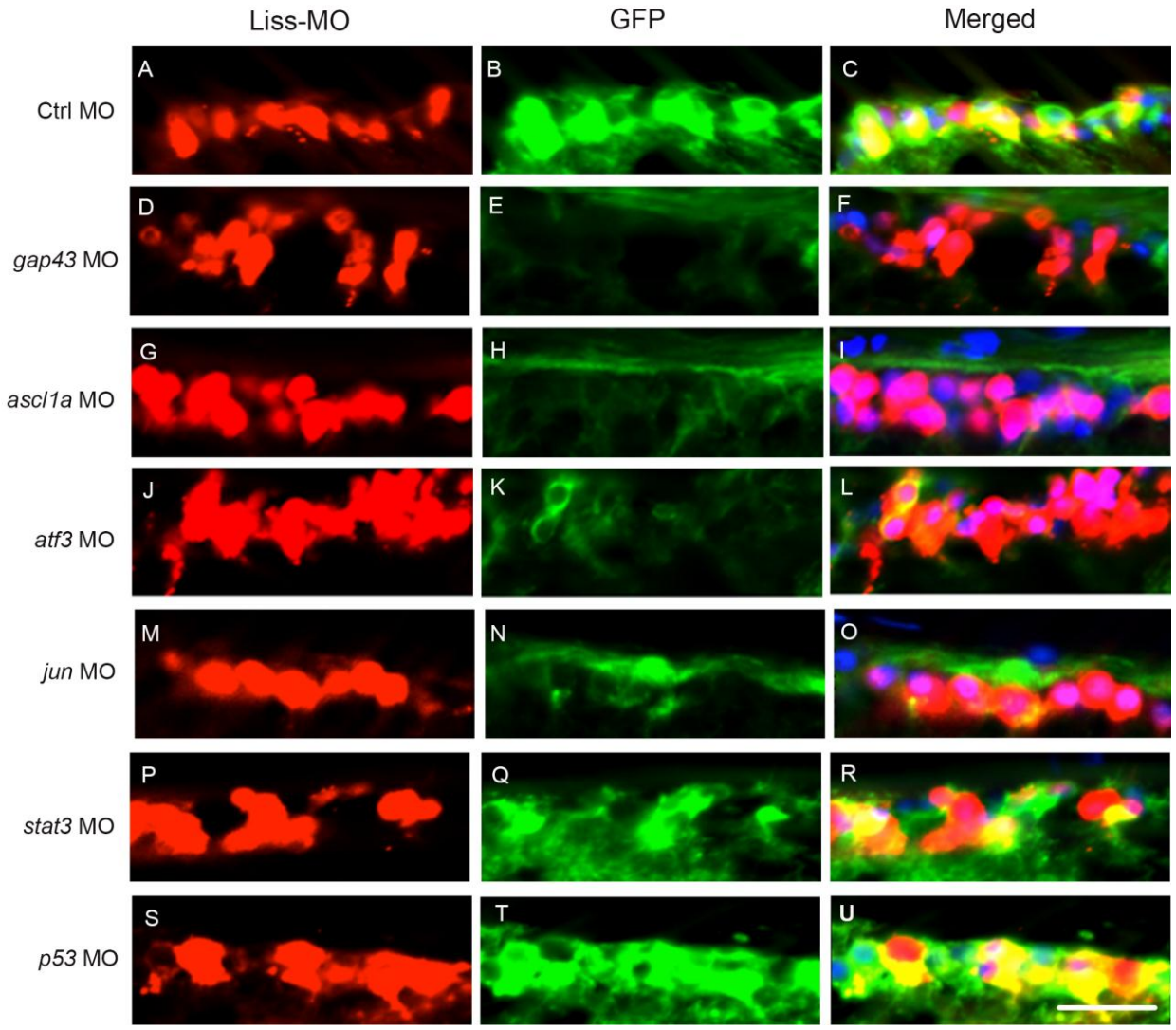


Fig. 13. Regeneration-associated transcription factors are differentially required for *gap43* reporter gene activation during optic nerve regeneration. Representative images of the RGC layer from transverse sections through regenerating adult retinas treated MO and preserved four days after optic nerve transection. Lissamine-labeled morpholinos (**red**) were delivered to RGCs through retrograde axonal transport after proximal optic nerve transection (**A, D, G, J, M, P,S**). The effect of MO-mediated gene knockdown on injury-induced *gap43* expression was assessed by *gap43*-GFP transgene

expression (**green**; **B, E, H, K, N, Q, T**). Expression of GFP in RGCs that have taken up the MO are visualized in the merged images (**yellow; C, F, I, L, O, R, U),** which include DAPI staining of nuclei in blue. Normally, RGCs express high levels of the *gap43*-GFP transgene in response injury as observed in samples treated with the negative control MO (**B, C).** As a positive control, RGCs treated with *gap43* MO show fully reduced *gap43-GFP* transgene expression as expected (**E, F).** In contrast, injury-induced *gap43*-transgene expression is greatly reduced in RGCs upon c-jun knockdown **(H, I)** , ATF3 knockdown **(K, L)** and Ascl1a knockdown **(N, O)**. RGCs treated with STAT3 MO show a partial reduction in *gap43*-GFP transgene expression **(Q, R)** p53 knockdown has no effects on *gap43*-transgene expression **(T,U)**Scale bar = 20 μm. n=6 for all treatment groups.

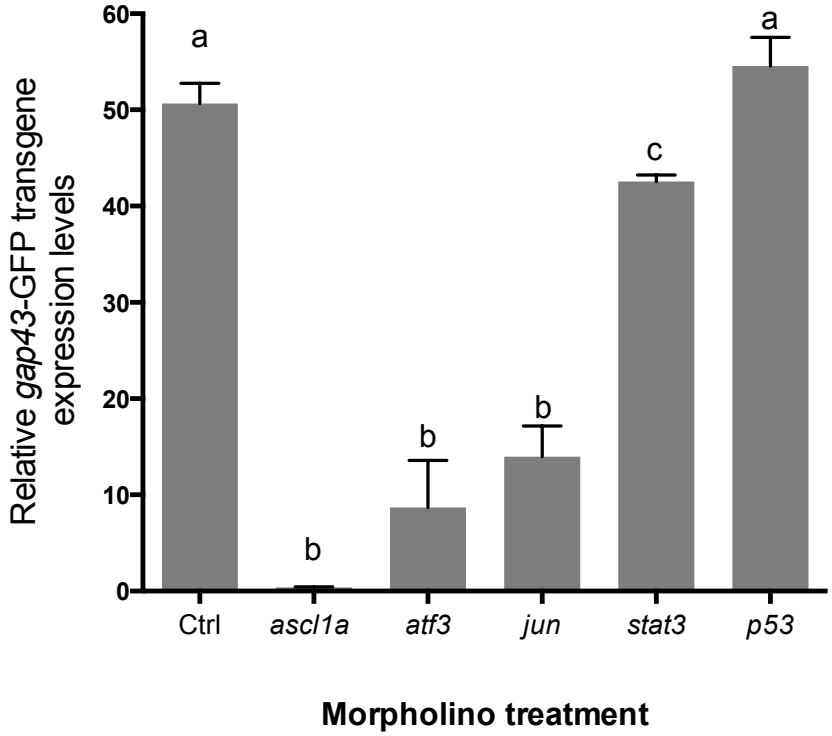


Fig. 14. Cooperative regulation of regenerative *gap43* reporter gene expression by a subset of regeneration-associated transcription factors. Total RNA extracts were prepared from control and regenerating retinas dissected four days after optic nerve transection and retrograde MO delivery. Relative fold change between uninjured and injured retina was determined by QPCR. Retinas treated with control MO show increased *gap43* reporter gene expression in response to optic nerve transection. In contrast, individual knockdown of Ascl1a, Atf3 or cJun leads to a significant reduction in injury-induced *gap43* reporter gene expression (b, $p<0.0001$). Knockdown of Stat3 partially reduces *gap43* reporter gene expression (c, $p<0.001$) and p53 knockdown has no effect on injury-induced transgene induction (a, not significantly different from control). Relative fold changes upon knockdown of Ascl1a (b), Atf3 (b) and cJun (b) are not statistically significant from each other but are statistically different from levels upon Stat3 knockdown (c) Statistical analysis: one-way ANOVA with Tukey post-hoc test. n=14 for control MO treated retinas, n=4 for each TF knockdown, error bars = SEM.

Stat3 knockdown reduces induction of *gap43* reporter expression, the effect is significantly different from *ascl1a*, *atf3* or *jun* knockdown (Fig. 14). Also consistent with the imaging analysis, we observed no significant effects on injury-induced *gap-43* reporter gene expression upon knockdown of p53 (Fig .14). We conclude that *ascl1a*, *atf3* and *jun* expression are essential for promoting *gap43* regeneration-associated gene expression in regenerating retinal ganglion cells, and that *stat3* contributes to the maximal levels of *gap43* expression.

Regulation of injury-induced *gap43* expression is evolutionarily conserved

Although fugu and zebrafish diverged over 300 million years ago, we previously demonstrated that the fugu *gap43* promoter is functionally conserved and promotes reporter gene expression in same spatial and temporal manner as the endogenous zebrafish *gap43* gene (Udvadia, 2008). However, since the genomic region upstream of the zebrafish *gap43* remains recalcitrant to sequencing efforts, we were unable to determine the level of sequence conservation in the *gap43* promoter regions between the two fish. Thus, it was necessary to determine if the effects of candidate transcription factor knockdown on the endogenous *gap43* gene were comparable to those we observed with the reporter gene.

We used QPCR to test the effects of candidate transcription factor knockdown on the regeneration-associated expression of endogenous zebrafish *gap43* gene. As previously reported, optic nerve injury leads to significant upregulation of endogenous *gap43* gene transcription, which is what we observed in RGCs treated with the control MO (Fig. 15). Knockdown of the individual transcription factors had effects similar to those we reported above on the reporter gene. As with the reporter gene, we observed that *ascl1a*, *atf3* and *jun* knockdown resulted in substantial reduction of *gap43* expression (8-16 fold reduction), while *p53* knockdown had no effect on *gap43* expression (Fig. 15). *Stat3*

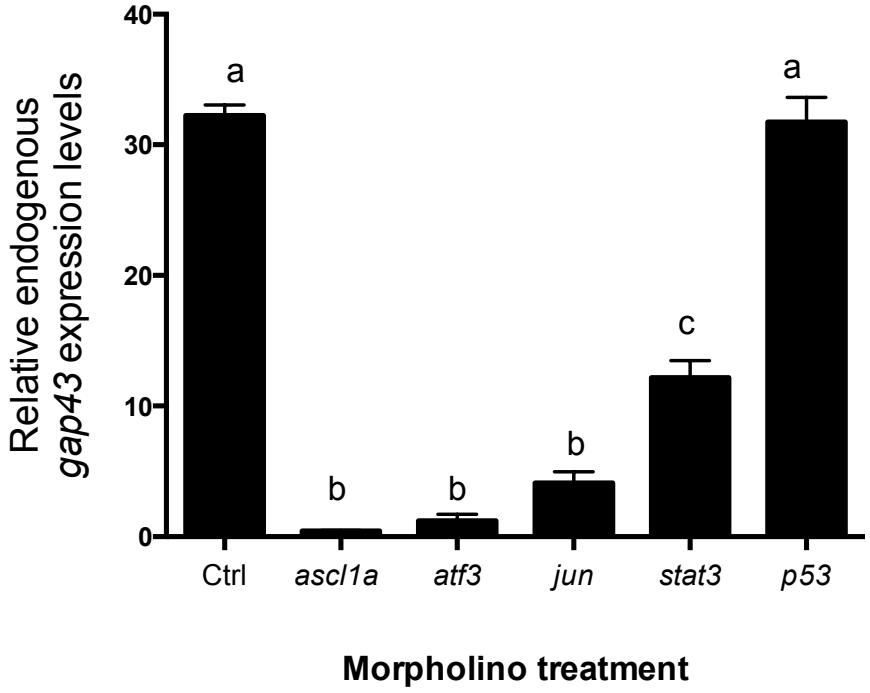


Fig. 15. Transcription factors regulating regeneration-associated *gap43* gene expression are functionally conserved between highly divergent teleost species. Total RNA extracts were prepared from control and regenerating retinas dissected four days after optic nerve transection and retrograde MO delivery. Relative fold change between uninjured and injured retina was determined by QPCR. Retinas treated with control MO show increased endogenous *gap43* expression post optic nerve transection. In contrast, individual knockdown of Ascl1a, Atf3 or cJun leads to significant reduction in endogenous *gap43* expression (b = $p<0.0001$). Knockdown of Stat3 partially reduces endogenous *gap43* expression (c = $p<0.001$) and p53 knockdown has no significant effect on endogenous *gap43* induction (a, not significantly different from control). Relative fold changes upon knockdown of Ascl1a (b), Atf3 (b) and cJun (b) are not statistically significant from each other but are statistically different from levels upon Stat3 knockdown (c). Statistical analysis: one-way ANOVA with Tukey post-hoc test. n=16 for control MO treated retinas, n=4 for TF knockdown, error bars = SEM

knockdown had significantly less of an impact on *gap43* expression than knockdown of *ascl1a*, *atf3* or *jun*, reflecting the same trend we observed with the reporter gene (Fig. 15). However, one difference we observed was that *stat3* knockdown appeared to have a more substantial effect on endogenous *gap43* expression (2-fold reduction, see Fig. 15) than on the reporter gene expression (20% reduction, see Fig. 14). We conclude that the transcription factors regulating regeneration-associated *gap43* expression are evolutionarily conserved, and that Ascl1a, Atf3, and cJun, act in a synergistic fashion to promote expression.

Ascl1a, Atf3 and cJun do not transcriptionally regulate each other during optic nerve regeneration

Based on our finding that knockdown of any one of the three transcription factors, *ascl1a*, *atf3*, or *jun* severely impacted regenerative *gap43* expression, we tested the possibility that these transcription factors transcriptionally regulated each other in response to optic nerve injury. We used QPCR to compare the expression of each transcription factor in regenerating retinas after uptake of MOs targeting either of the other two transcription factors. We found no significant differences in injury-induced expression of *ascl1a*, *atf3*, or *jun* under any of the conditions (Fig. 16)**,** suggesting that regenerative expression of transcription factors Atf3, Ascl1a and cJun are not dependent on each other. This finding leaves open the possibility that these transcription factors work cooperatively, by either forming complexes, or by otherwise facilitating promoter binding to regulate regeneration-associated *gap43* expression.

Transcription factors necessary for re-establishment of retinotectal projections after optic nerve injury

We demonstrated that previously identified regeneration-associated transcription factors, Ascl1a, Atf3, cJun, Stat3, and p53 have differential activity with regard to regulating

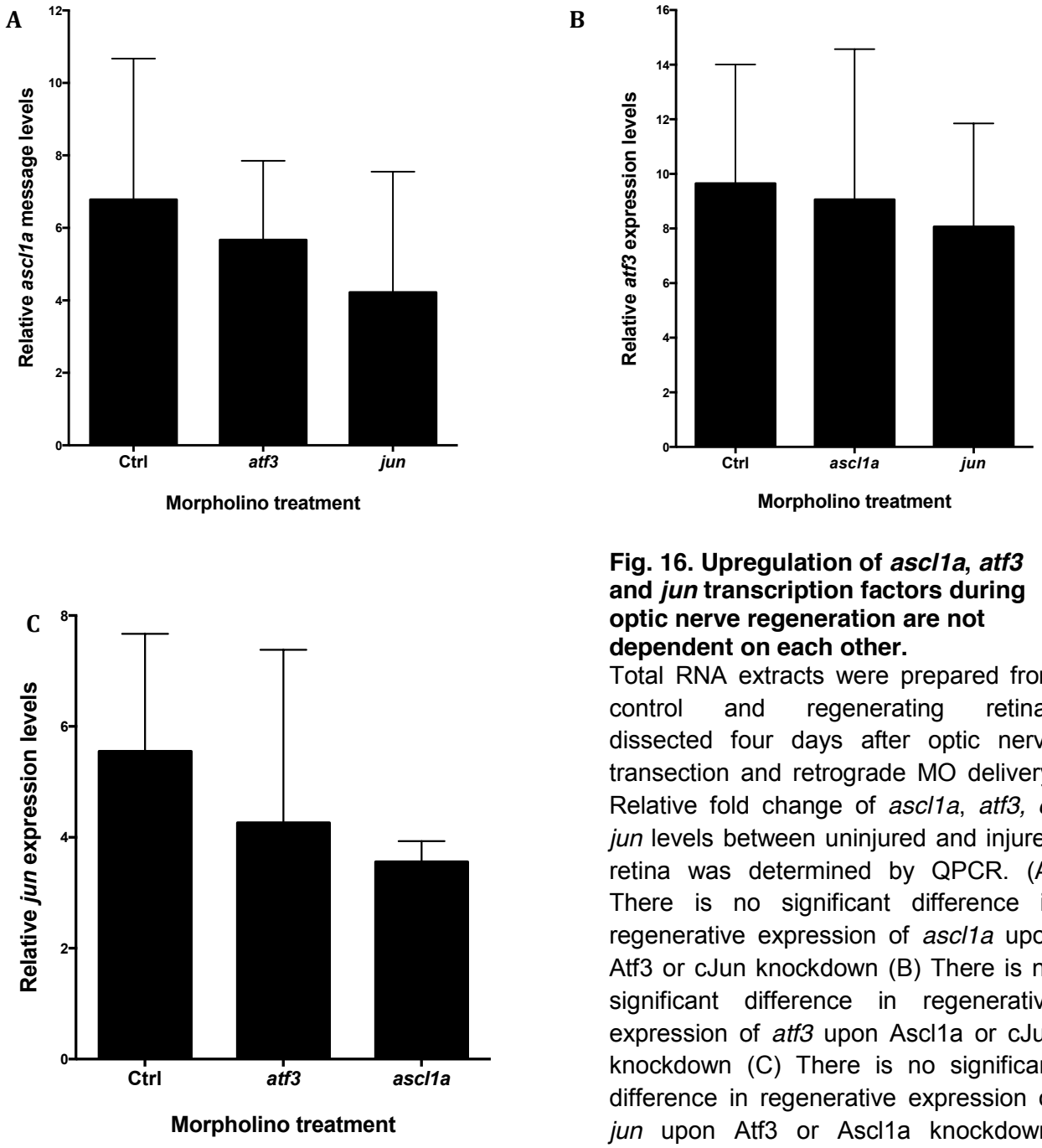


Fig. 16. Upregulation of *ascl1a*, *atf3* and *jun* transcription factors during optic nerve regeneration are not dependent on each other.

Total RNA extracts were prepared from control and regenerating retinas dissected four days after optic nerve transection and retrograde MO delivery. Relative fold change of *ascl1a*, *atf3, or jun* levels between uninjured and injured retina was determined by QPCR. (A) There is no significant difference in regenerative expression of *ascl1a* upon Atf3 or cJun knockdown (B) There is no significant difference in regenerative expression of *atf3* upon Ascl1a or cJun knockdown (C) There is no significant difference in regenerative expression of *jun* upon Atf3 or Ascl1a knockdown. Statistical analysis: one-way ANOVA p value = 1 (Not significant), error bars = SEM. n=4 for each

gap43 expression after optic nerve injury. We next wanted to determine how the ability to ability of these transcription factors to regulate *gap43* expression correlated with their overall ability to promote successful CNS axon regeneration. Given that each of these transcription factors is likely to regulate additional regeneration-associated genes, we hypothesized that a transcription factor that did not regulate *gap43* expression, such as p53, could still have the potential to impact overall regeneration. To address this possibility, we used the same method outline in Fig.9 to test how MO-mediated knockdown of *ascl1a*, *atf3*, *jun*, *stat3*, or *p53* affected retinotectal regeneration.

Our results demonstrate a direct correlation between the ability to promote regenerative expression of *gap43* and the ability to promote retinotectal regeneration (Fig. 17). We observed that knockdown of *ascl1a, atf3, and jun* nearly prevented regeneration altogether, while knockdown of *stat3* caused a partial reduction in regeneration, and knockdown of *p53* had no effect on regeneration. We quantified the results by calculating the percent regeneration as described above. We found that after *ascl1a* or *atf3* knockdown, which nearly abolished regenerative *gap43* expression (Fig. 15), only 1-3% of injured neurons show complete axon re-growth to the tectum (Fig. 18). This is similar to the percentage of RGCs that regenerated after *gap43* knockdown (Fig. 11). Knockdown of *jun* permitted only 20% regeneration, while *stat3* knockdown still allowed 30% regeneration compared to the 50-60% regeneration observed with the control MO (Fig. 18). Finally, similar to its effects on *gap43* expression, knockdown of *p53* did not significantly impact axon re-growth to the tectum (Fig. 18). These results identify *gap43* is a significant regeneration-associated target of Ascl1a, Atf3, cJun, and Stat3, and demonstrate that the ability to upregulate *gap43* expression after CNS injury clearly correlated with successful CNS regeneration.

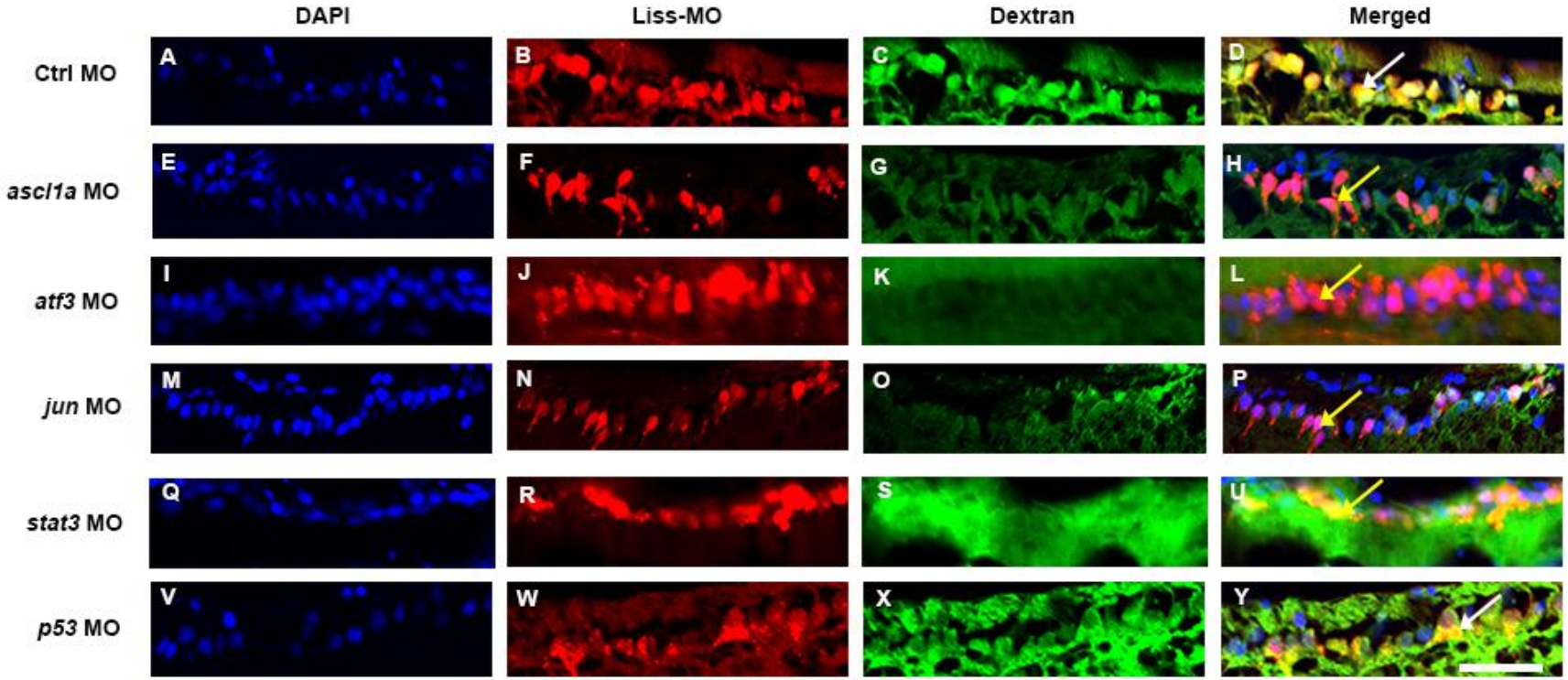

DAPI
Liss-MO
Dextran
Merged
Ctrl MO
ascl1a MO
atf3 MO
jun MO
stat3 MO
p53 MO
A
B
C
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Fig. 17. Ability of candidate transcription factors to promote regeneration is correlated with their ability to promote regenerative *gap43* expression. Representative images of the RGC layer from transverse sections through regenerating adult retinas treated with control MO (B) or morpholinos targeting candidate transcription factors (F, J, N, R, W). Lissamine-labeled morpholinos (red) were delivered to RGCs through retrograde axonal transport after proximal optic nerve transection (B, F, J, N, R, W). RGC axons regenerating to the tectum were retrogradely labeled with fluoresceinated dextran (green) four days post-injury (C, G, K, O, S, X). Retinas were harvested 9 days after tectum labeling, cryosectioned and coverslipped with DAPI mounting medium to stain the nuclei (blue). Control MO treated RGCs displayed robust re-growth as evident by the co-localization of MOs and tracer (D, white arrow). In contrast, few RGCs regenerated in retinas treated with *ascl1a* MO (H), *atf3* MO (L), *jun* MO (P) and *stat3* MO (U) as evident by lack of co-localization with tracer (yellow arrow). Scale bar = 20 μM

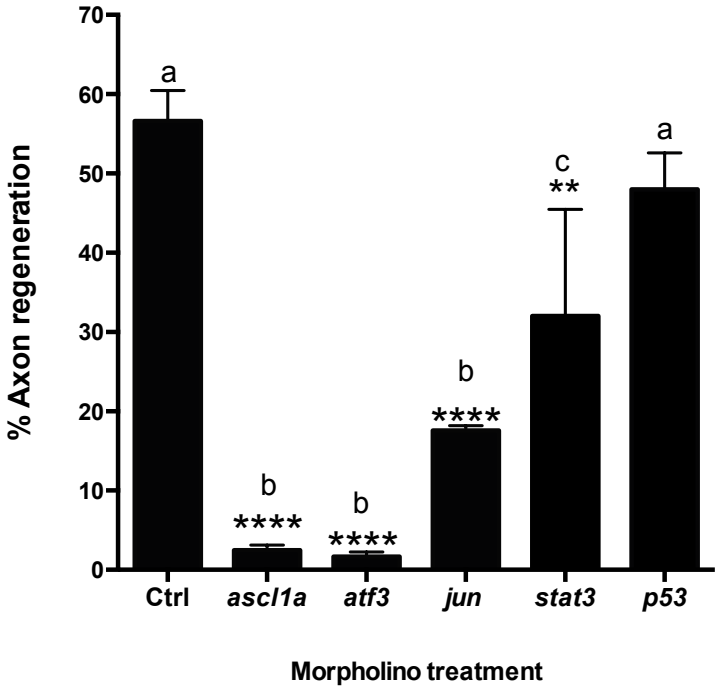

Fig. 18. Expression of *ascl1a* and *atf3* are required for re-establishment of retinotectal projections after optic nerve transection. Quantification of the percentage of RGCs that received the MOs and were able to regenerate axons to the optic tectum. Compared to controls (n=9), RGCs receiving *ascl1a* and *atf3* MO show (n=4) exhibited significantly reduced optic nerve regeneration. RGCs treated with *jun* and *stat3* MO display reduced regeneration compared to controls. *p53* MO treated RGCs regenerated to the same extent as controls. (**=p<0.01,****=p<0.0001). Percent axon regeneration upon *ascl1a* (b), *atf3* (b) and *jun* (b) knockdown are not different from each other but are different from *stat3* knockdown (c). Statistical analysis: ONE- way ANOVA with Tukey post hoc. n=3- 4 per treatment group, error bars = SEM

Conclusions and summary:

Using a combination of *in vivo* regeneration assays and *in vivo* reporter assays, we have identified specific transcriptional complexes underlying successful CNS regeneration in fish. We first demonstrated that Gap43 expression in response to optic nerve injury is *necessary* for successful re-innervation of transected RGC axons in the tectum. We have next used Gap43, as a probe to identify transcriptional regulatory pathways modulating gene expression and axon outgrowth in response to CNS injury.

Previously, we have utilized the pufferfish *gap43* promoter as a proxy to endogenous zebrafish *gap43* gene to identify promoter regions indispensable for initiation of *gap43* expression in response to optic nerve injury. Current work extends these findings by revealing specific transcription factor complexes required in re-initiating *gap43* expression upon CNS injury. Induction of regenerative *gap43* expression in response to CNS injury is dependent on transcription factors Ascl1a, Atf3, cJun and Stat3. The function of these transcription factors in driving regenerative *gap43* expression is well conserved between highly divergent teleost species. We also observe that knockdown of any one of the transcription factors, Ascl1a, Atf3 or cJun, appears to greatly diminish regenerative *gap43* expression and correspondingly axon regeneration. This finding reveals the cooperative action of Ascl1a, Atf3 and cJun in driving regenerative *gap43* expression in fish, the implications of which are discussed in further detail in the next chapter. Together, these findings enhance our understanding of transcriptional pathways underlying successful CNS regeneration, revealing potential pathways that could be targeted to improve CNS regeneration in mammals.

CHAPTER IV

General Discussion

Summary of key findings

Using a combination of *in vivo* reporter and regeneration assays, we have identified gene regulatory pathways underlying successful CNS regeneration in fish. We show that *gap43* re-expression post-injury is critical for RGCs axons to re-establish tectal connections following optic nerve transection. We demonstrate that Gap43 expression during regeneration is regulated by the transcription factors Ascl1a, Atf3, cJun and Stat3. Furthermore, we show that the impact of these transcription factors on axon outgrowth is proportional to their impact on regenerative *gap43* expression. In particular, Ascl1a, Atf3 and cJun are essential and appear functionally intertwined in their requirement for initiating regenerative *gap43* expression in a manner that is evolutionarily conserved across teleost evolution. These results reveal a cell intrinsic mechanism that regulates the ability of CNS neurons to switch to a growth state and mount a functional regenerative response. The significance of these results in the context of the broader CNS regeneration field and the future directions in which they may lead are discussed below.

Growth associated protein-43 in CNS regeneration

Gap43 is a growth-associated protein heavily enriched in growth cone membranes during nervous system development and regeneration (Skene*., 1*989, Benowitz and Routenberg, 1997). Expression of Gap43 is augmented during peripheral nervous system regeneration (Chong *et al*., 1992) and certain types of CNS injuries (Sommervaille *et al*., 1991). Conceptually, most research on Gap43 has focused on testing whether forced expression of Gap43 is sufficient to enhance CNS regeneration in mammalian neurons, that normally fail to mount a successful regenerative response (Bomze *et al*., 2001 , Zhang *et al*., 2005). In contrast, we have addressed the question of whether Gap43 is necessary for successful CNS regeneration.

We observe that in the absence of Gap43, there is a dramatic reduction in the number of RGC axons successfully re-innervating the optic tectum. Our findings demonstrate that re-expression of Gap43 following injury is necessary and critical for successful CNS regeneration. In this study, we have focused on identifying the effects of Gap43 knockdown on initial axon outgrowth to the tectum. The impact of Gap43 knockdown 30 and 60 days post-injury, correlating with ensuing stages of regeneration involving synaptic refinement are important questions to study in the future. The main focus of this work is transcriptional regulation of Gap43 and corresponding effects on axon regeneration. However, Gap43 function is also regulated at the level of post-translational modifications such as phosphorylation. To fully understand the contributions of Gap43 to successful

CNS regeneration, it will also be necessary to understand how post-translational regulation of Gap43 affects axon re-growth.

Gap43 is a major substrate of protein kinase C (PKC) in axonal growth cones and phosphorylation by PKC impacts Gap43 localization and its interactions with other proteins. Phosphorylated Gap43 is localized to areas of the growth cone membranes that are engaged in productive interactions with the substrate (Dent and Meiri, 1992). In contrast, unphosphorylated Gap43 is localized to regions of the growth cone that are retracting (Dent and Meiri, 1992). Constitutively phosphorylated GAP43 promotes f-actin-regulated filopodial formation, whereas unphosphorylated Gap43 modulates microtubule dynamics (Nguyen *et al*., 2009). Since the capacity of growing neurons to respond to directional pathfinding cues is critically dependent on the balance of microfilament and microtubule dynamics in the growth cones (Bouquet and Nothias 2007, Pak *et al* 2008, Nguyen *et al*., 2009), regulation of Gap43 phosphorylation is thought to function by modulating axon growth and guidance in response to external cues in the developing landscape. However, its role in the regenerating landscape is less clear.

In fish, during optic nerve regeneration, there is a biphasic pattern of phosphorylated gap43 mRNA and protein correlating with key regenerative phases (Kaneda *et al*., 2008). The initial peak in phosphorylated Gap43 at 4-10 days following injury corresponds to the increase in axon re-growth to the tectum.

The second peak 30-80 days post injury corresponds to synaptic refinement of retinotectal topography (Kaneda *et al*., 2008). In transient transgenic experiments in developing zebrafish, Gap43 phosphorylation was shown to be critical for the growth and branching of retinotectal arbors. Thus, it is possible that the regulation of Gap43 phosphorylation in retinal axons post-injury is also likely to impact target re-innervation and subsequent synaptic refinement.

In our lab, we have generated stable transgenic zebrafish lines that can be induced to express wild type *gap43* or a mutant form of *gap43* (*gap43S42A*) that cannot be phosphorylated by PKC (Forecki and Udvadia, *in preparation*). These lines allow temporal control in the induction of wild type and mutant Gap43. Inducing the mutant protein at various stages of retinal ganglion cell development causes axon growth and guidance defects in the formation of retinotectal connections. Recent evidence has emerged that suggests that under conditions that permit mammalian optic nerve regeneration, re-growing RGC axons often show mistargeting and misguidance (Pernet and Schwab, 2014). This suggests the importance in understanding the role of axonal guidance cues and how they are transduced intracellularly to ensure successful target re-innervation. Future studies using the inducible *gap43* S42A zebrafish line could be used to address the importance of Gap43 phosphorylation in transducing guidance signals that lead to successful retinotectal regeneration.

Candidate transcription factors – Role in driving regenerative gap43 expression and CNS regeneration

Previously we identified regeneration specific DNA elements within the fugu *gap43* promoter containing putative binding sites for several regeneration-associated candidate transcription factors such as Ascl1a, Atf-3, cJun, Stat3 and p53 among others (Kusik *et al*., 2010). Notably, all of these transcription factors were shown to be induced several fold following optic nerve transection in zebrafish (Veldman *et al*., 2010), making them strong candidates for transcriptional regulation of regenerative *gap43* expression. We performed gene knockdown experiments to test the hypothesis that these candidate transcription factors are required for *gap43* re-induction after injury. Our objective was to utilize *gap43* as a model to understand regulatory pathways underlying successful regeneration in fish. In the following sections I will elaborate on the key findings and relevance for each candidate transcription factor with respect to regulation of growth-associated gene expression and CNS regeneration.

cJun

The AP-1 transcription complex regulates neural development and consists of homo- or hetero-dimeric complexes between members of the Jun, Fos, and ATF/CREB families (Smita and Patodia., 2012). *Jun* is activated as an immediate early gene (IEG) following nerve injury and persists at high levels in injured neurons during the entire peripheral regenerative process (Herdegen et al., 1991; Kenney and Kocsis, 1998; Mason et al., 2003; Raivich et al., 2004; Lindwall and

Kanje, 2005; Ruff et al., 2012). In a facial nerve transection model, neuronal deletion of *jun* results in strongly reduced target innervation and delayed functional recovery, concomitant with a loss of Cd44, galanin and integrin expression (Raivich *et al*., 2004). In the mammalian CNS, overexpression of *jun* leads to cortical neurite outgrowth (Lerch *et al*., 2014).

Surprisingly, the forced expression of *jun* in cortical slices did not lead to increases in expression of Gap43 or Integrin alpha 7, both considered potential downstream targets of JUN (Lerch *et al*., 2014, Schaden *et al*., 1994, Weber and Skene, 1998, Ekstrom *et al*., 2003). In contrast, our results show that during successful CNS regeneration in fish, cJun is required for inducing regenerative *gap43* expression. While this may reflect a species-specific difference, we speculate that forced expression of cJun in cortical slices alone is not sufficient to induce *gap43* expression because the neurons are still lacking the proteins that heterodimerize with cJun to form transcriptional activators. cJun homodimers have a lower affinity for AP-1 promoter sites compared to cJun/cFos or cJun/Atf3 heterodimers (Halazonetis *et al*., 1988). In addition, Overexpression of cJun might result in the formation of aberrant homodimeric transcriptional complexes that result in transcriptional repression. Thus, it is possible that cJun has dual functions during axon regeneration. cJun homodimers may be necessary for repressing genes that inhibit regeneration, while cJun heterodimers may be necessary for activating genes the promote axon growth.

We observe that knockdown of *jun* results in a significant reduction in the number of severed axons re-growing to the optic tectum 2 weeks following injury. This reduction in axon regeneration is proportional to the ability of cJun to drive regenerative *gap43* expression. Thus, cJun has a conserved role in driving axon growth across species and is a critical component of successful CNS regeneration.

Atf3

Atf3 belongs to the bZIP family of transcription factors, and forms heterodimeric interactions with bZIP transcription factors from the AP-1 and CEBP families (Hai and Curran, 1991; Hai and Hartman, 2001). Atf3 is normally expressed at basal levels, but is rapidly induced by peripheral nerve injury in mammals (Tsujino et al., 2000; Raivich and Behrens, 2006; Hyatt Sachs et al., 2007; Zigmond and Vaccariello, 2007) and optic nerve injury in fish (Saul *et al*., 2010). Homodimeric Atf3 complexes primarily act as transcriptional repressors (Nakagomi et al 2003, Chen et al 1996, Hai and Hartman, 2001). In contrast, the heterodimeric interactions of Atf3 and cJun result in transcriptional activation (Nakagomi et al 2003, Hsu et al., 1992; Hai and Hartman, 2001). *In vitro*, expression of Atf3 along with cJun enhanced cJun-mediated neurite sprouting (Pearson *et al*., 2003). *In* vitro, Atf3 interactions with cJun were also important in initiating a signaling cascade involving upregulation of *heat shock protein 27* (*hsp27*) and Akt activation leading to neurite elongation (Nakagomi *et al*., 2003). Transgenic mice

constitutively overexpressing Atf3 in uninjured DRG neurons show enhanced rates of peripheral regeneration, comparable to the rates observed with a pre-conditioning nerve injury (Seijjfers *et al*., 2007). This enhancement in peripheral nerve regeneration was accompanied by a modest increase in expression of regeneration associated gene Sprr1a, but not Gap43 (Seijjfers *et al*., 2007).

In fish, two independent studies have identified *atf3* as one of the few genes showing >20 fold increase in expression 24 hours post optic nerve injury (Veldman *et al*., 2007, Saul *et al., 2*010). We observe that upon *atf3* knockdown in regenerating RGCs, *gap43* expression is dramatically reduced, confirming that *atf3* expression is required for inducing regenerative *gap43* expression. The Atf3 protein is up regulated in response to optic nerve injury in both mammals and fish. One possibility is that the downstream gene targets activated by Atf3 account for the differential ability of animals to regenerate the optic nerve. Consistent with this theory, we observe that Atf3 upregulation post-injury initiates *gap43* transcription in fish, whereas similar *gap43* re-induction was not observed when Atf3 was constitutively overexpressed in mammals (Sejjfers *et al*., 2007). One explanation for this difference is that constitutive overexpression of Atf3 may encourage Atf3 homodimer formation that abrogates gene activation. Thus, it may be that it is not simply the presence of Atf3 that is necessary to promote axon regeneration. Rather, key regeneration-associated gene activation may require the proper stoichiometry of Atf3 and other bZIP proteins for appropriate heterodimerization. Consistent with this interpretation, we observe that either *atf3*

or *jun* knockdown dramatically reduces optic nerve regeneration *in vivo* at levels proportional to the impacts on regenerative *gap43* expression.

Stat3

Signal transducer and activator of transcription 3 (Stat3) is a well-studied transcription factor regulating several cellular processes including migration, proliferation, apoptosis and immune responses (Levy and Lee, 2002). Axonal Stat3 activated at the injury site has dual roles: 1) in retrograde injury signaling and 2) as a transcription factor that initiates regeneration associated gene transcription (Patodia and Raivich *et al., 2*012). We demonstrate here that Stat3 is necessary for driving expression of an important regeneration-associated gene *gap43.* Also, effects of Stat3 knockdown on regeneration *in vivo* is proportional to the effects on *gap43* expression.

Our work complements studies that demonstrate that over expression of constitutively active forms of Stat3 is sufficient to initiate neurite outgrowth in neurons that are normally incapable of functional regeneration. In a transcriptional profiling study of intrinsic PNS factors in the postnatal mouse, Stat3 was found to be constitutively enriched in the PNS and was shown to enhance neurite outgrowth of cerebellar granule neurons *in vitro* (Smith *et al*., 2011). In adult mouse RGCs, a constitutively active form of Stat3 increased axon outgrowth following injury, although pharmacological blockage of Rho/ROCK

pathway was required to prevent guidance errors observed in the regenerating axons (Pernet *et al*., 2013). Miao *et al., 2*006 demonstrated a requirement for Stat3 in the neurite outgrowth of rat primary sensory neurons. Baryere *et al*., 2011 through time-lapse fluorescence microscopy demonstrated that Stat3 promotes neurite outgrowth following PNS lesion, but seems to possess a phase-specific role in promoting outgrowth. Specifically, the authors observed that Stat3 is crucial for initiation of outgrowth, but not necessarily for sustaining axon growth (Baryere *et al*., 2011). Altogether these studies along with our findings confirm that Stat3 is an important mediator of regeneration and further downstream targets, in addition to *gap43* remain to be identified.

There is considerable evidence that suggests redundant roles for Stat3 and cJun in modulating axon regeneration. Patodia and Raivich *et al*., 2012 observed that in *stat3* conditional knock out mice, expression of key regeneration-associated genes such as CD44, β1 integrin, CGRP, and galanin, as well as nuclear transport of Atf3 was reduced or abolished in some cases. Interestingly, cJun expression was unchanged in Stat3 conditional knock out mice. Considering Stat3 and cJun share some gene targets (Raivich *et al*., 2004, Patodia and Raivich., 2012), one can speculate that cJun and Stat3 have overlapping functions in promoting regeneration. The functional overlap with cJun could explain our finding that Stat3 knockdown does not impact either regenerative *gap43* expression and axon regeneration to the same extent as the knockdown of other transcription factors.

Recently Jak/Stat signaling was also identified to mediate successful optic nerve regeneration in fish (Elsaedi *et al*., 2014). Surprisingly, in addition to stimulating axon growth-associated genes, Jak/Stat signaling also induced the expression of well-characterized negative regulators of axon regeneration namely Socs3 and Sfpq. While CNS regeneration proceeds in the zebrafish even in the presence of such inhibitory factors, knockdown of Socs3 and Sfpq leads to enhanced regeneration. The finding that CNS injury in fish induces similar negative modulators of axon regeneration to those observed in mammals is significant because it further validates the use of zebrafish as an appropriate model for vertebrate CNS regeneration. Understanding how fish overcome these common inhibitory signals to mount a successful regenerative response will be directly relevant to discovering therapeutics for human CNS nerve injuries.

Ascl1a

The basic helix-loop-helix (bHLH) transcription factor, Mash1 (aka Ascl1), is transiently expressed during the development of many neuronal subtypes and directly regulates genes involved in axon growth (Tomita et al., 1996, Johnson et al., 1990,Castro et al., 2011). In fish, Ascl1a is one of the earliest genes to be induced upon optic nerve injury and was shown to promote neurite outgrowth in dissociated retinal ganglion cells (Fausett *et al*., 2008). Although Ascl1a was

demonstrated to promote RGC neurite outgrowth in vitro, a direct role for Ascl1a in axon regeneration in vivo was not previously demonstrated.

Ascl1a has been shown to be necessary in a different aspect of retinal regeneration that occurs in response to photoreceptor damage. In addition to regenerating damaged CNS axons, fish are also capable of stimulating neurogenesis in response to chemical or light damage of photoreceptors to replace the neurons lost due to cell death. This is accomplished by Mueller glia, which de-differentiate and form multipotent retinal progenitors (Raymond et al., 2006; Bernardos et al., 2007; Fausett et al., 2008; Thummel et al., 2008; Ramachandran et al., 2010). In fish, expression of *ascl1a* was shown to be critical for reprogramming differentiated Mueller glia into actively dividing retinal progenitors (Fausett *et al*., 2008). Interestingly, Stat3, which has established roles in axon regeneration was shown to be necessary for maximal *ascl1a* expression and proliferation of Mueller glia during photoreceptor regeneration in fish (Nelson *et al*., 2012). In addition, *tuba1a* and *pax6a*, which are upregulated in response to axonal injury, are also downstream targets of Ascl1a in Mueller glia undergoing injury induced de-differentiation and proliferation (Fausett *et al*., 2008, Ramachandran *et al., 2*010, Ramachandran *et al*., 2012). Thus photoreceptor cell death and optic nerve damage both elicit the Ascl1a pathway as part of two distinct regenerative responses involving neurogenesis and axon growth.

Given that the *ascl1a* gene was shown to be induced by optic nerve injury and promote neurite outgrowth in cultured RGCs, we investigated the ability of Ascl1a to initiate regeneration-associated gene transcription of *gap43* and to promote target reinnervation after optic nerve transection. Previously, Fausett *et al*., 2008 showed that Ascl1a induced regenerative expression of another regeneration-associated gene, *tuba1a*, in fish after retinal injury. This induction was dependent on an E-box motif in the *tuba1a* promoter (Fausett *et al*., 2008). Promoter analysis of the fugu *gap43* promoter has identified a similar teleost conserved E-box motif (Kusik *et* al., 2010). We show that Ascl1a is essential for *gap43* expression during optic nerve regeneration, and that Ascl1a knockdown also greatly reduces axon re-growth to the tectum following optic nerve transection.

Although the mammalian homologue of Ascl1a (Mash1) is not normally expressed upon photoreceptor cell death or RGC axotomy, forced expression of Mash1 in mammals has similar effects to the injury-induced Ascl1a expression in fish. For example, virally-mediated ectopic expression of Mash1 in either dissociated Mueller glial cultures or intact mouse retina was sufficient to induce neurogenesis (Pollack *et al*., 2013). Gene expression analysis of Mash1-infected Mueller glia showed the activation of retinal progenitor genes and concomitant downregulation of glial genes. Furthermore, in Mash1-infected cells the chromatin landscape surrounding retinal progenitor genes was converted from a repressive to an active state, reprogramming Mueller glia to a proliferating state to replace dying neurons after retinal injury.

To investigate whether forced expression of Mash1 would improve axon regeneration after spinal cord injury, our collaborators ectopically expressed Mash1 in noradrenergic brainstem neurons of adult rats (Williams *et al*., submitted). After complete transection of the thoracic spinal cord and implantation of a Schwann cell (SC) bridge, Mash1 led to increased noradrenergic axon regeneration into the SC bridge. In addition, axon regrowth was correlated with eventual partial recovery of locomotor function in the injured animals, supporting the therapeutic value of such an approach. Taken together, the Ascl1a knockdown studies in the fish and the Mash1 forced expression studies in mammals provide compelling evidence for an evolutionarily conserved role for Ascl1a/Mash1 in promoting regeneration through both neurogenic and axonogenic pathways.

p53

p53 is a member of a family of tumor suppressors and is known to carry out both pro-apoptotic and anti-apoptotic roles in the nervous system (Culmsee and Mattson, 2005, Jacobs et al., 2006). In addition, *in vitro* studies have demonstrated a role for p53 in inducing neurite outgrowth in PC12 cells, in part by regulating regeneration-associated genes *Coro1b* and *Rab13* (Di Giovanni et al., 2006). Furthermore, in the PNS, which normally exhibits a robust regenerative response, p53 knockout mice display a significant decrease in the number of fibers re-innervating target muscles following facial axotomy (Di

Giovanni et al., 2006). In mammals, a novel transcriptional complex formed by acetylated p53 and the acetyltransferases CBP/p300, was recruited to the *gap43* promoter *in vivo* following facial nerve transection thereby driving regenerative *gap43* and axon regeneration *in vivo* (Tedeschi *et al*., 2009).

Surprisingly, our results show that p53 is not required for *gap43* induction during optic nerve regeneration in fish. In contrast to findings that show that both *gap43* expression and axon regeneration in mice after facial nerve transection is dependent on p53 activity, we found that p53 knockdown had no significant effect on *gap43* expression or target reinnervation after optic nerve transection in fish. (Tedeschi *et al*., 2009). These apparent differences in the requirement for p53 in promoting regenerative *gap43* expression and regenerative axon growth could be due to at least two differences in experimental design. One possibility is that p53-dependent *gap43* expression is a species-specific phenomenon. If this is the case it is not due to the lack of p53 binding sites within the teleost *gap43* promoter (Kusik et al., 2010) or to the lack of p53 induction after nerve injury (Veldman et al., 2007). Thus, it is possible that in the fish there are redundant pathways activated in response to injury that can compensate for the lack of p53. A second possibility is that *gap43* is regulated differently after PNS injury and CNS injury. In support of differential regulation of growth associated genes, we have previously shown that activation of *gap43* expression in regenerating RGCs requires additional promoter elements from those require to activate expression in developing neurons (Kusik et al., 2010). The regulation of another regeneration-associated gene, *tuba1a*, was also shown to have different

requirements for transcription during development and regeneration (Goldman and Ding, 2000). Future experiments comparing the role of p53, and other regeneration-associated transcription factors in CNS and PNS regeneration paradigms within the zebrafish model will help to distinguish between these possibilities.

Combinatorial transcription factor regulation of regenerative *gap43* expression and axon outgrowth

One of the important findings from this work is evidence that Ascl1a, Atf3 and cJun act in a synergistic, rather than additive manner to promote transcription of *gap43* gene during CNS regeneration. Combinatorial regulation by transcription factors has often been touted as the basis by which cells use minimal cellular resources to elicit complex signaling cascades. Recently there has been a steady increase in studies demonstrating evidence for combinatorial transcription factor regulation of a variety of cellular processes. The list includes but is not limited to terminal differentiation of the dopaminergic nervous system in *C.elegans* (Doitsidou et al., 2013), regulation of ion channel gene expression (Wolfram *et* al., 2014), determination of cardiac cell fates (Junion *et al* ., 2012), odorant receptor expression in *D.melanogaster* (Jafari *et al*., 2012), and patterning of chromatin regulators in human cells (Ram *et al*., 2011). More importantly, axonal guidance, recently demonstrated to be critical factor in determining regenerative success of RGCs axons during mammalian optic nerve

regeneration (Pernet and Schwab *et al*., 2014), has also been demonstrated to be regulated by a combinatorial transcription factor network (Zarin *et al*., 2014).

A list of possible models for synergistic effects of candidate transcription factors is summarized in Fig.19. As previously discussed, we have tested and ruled out the possibility that candidate transcription factors regulate the expression of each other at the transcriptional level (Fig.16). In the context of axon regeneration, it is being increasingly recognized that manipulations with a single transcription factor or growth-associated gene rarely leads to robust re-growth confirming that successful regeneration is dependent on a multi-nodal transcription network that is highly connected rather than isolated (Tedeschi *et al*., 2012). There is plenty of evidence to suggest co-operative actions for Atf3 and cJun during axon regeneration (Pearson *et al*., 2003,Tsujino *et al*., 2000,Nakagomi et al 2003). In light of our current findings, it is not surprising that overexpression of Atf3 alone (Seijjfers et al 2007) or cJun alone (Learch et al 2014) in the mammalian CNS, did not lead to a corresponding increase in expression of *gap43.* Overexpression of either Atf3 or cJun alone, likely hampers heterodimer formation, which could even lead to transcriptional repression of target genes such as *gap43*. To test the possibility that cJUN and Atf3 heterodimerize to regulate key regeneration associated genes during zebrafish optic nerve regeneration (Fig.19), sequential chromatin immunoprecipitation on regenerating retinas may be performed. Interestingly, Ascl1a solely studied in the context of reprogramming Mueller glia into a proliferative state, is part of a cohort of transcription factors needed to re-

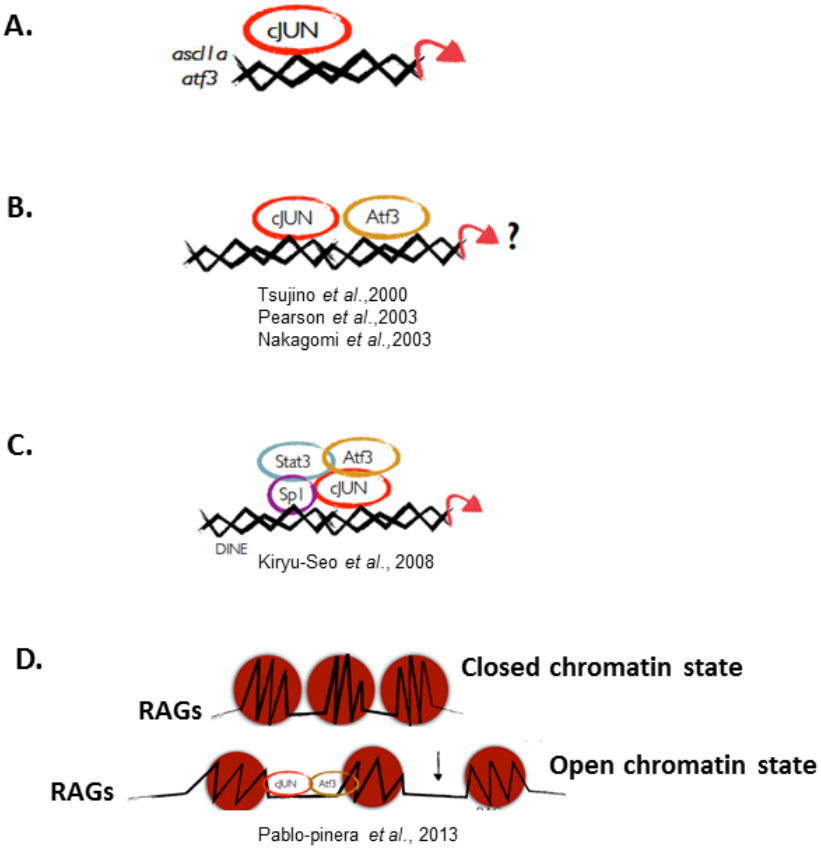

Fig. 19. Potential models for synergistic effects of candidate transcription factors. (A) Candidate transcription factors regulate each other at the level of transcription (B) Transcription factors cJUN and Atf3 heterodimerize to regulate key regeneration associated genes (C) Presence of anchor TF that creates a complex at the promoter of key regeneration associated genes (D) Simultaneous binding of multiple transcription factors leads to an open chromatin state thereby facilitating binding of other TFs due to increased DNA accessibility around promoters of key regeneration associated genes

activate regenerative *gap43* expression. Further studies are needed to clarify whether Ascl1a, Atf3 and c-Jun induce expression of Gap43, through direct promoter occupancy during CNS regeneration.

In addition to formation of heterodimeric complexes, Atf3 and cJun can also interact with other transcription factors such as Stat3, through utilization of co-activators or by synergizing with transcription factors that bind to adjacent DNA binding sites. *In vitro* and *in vivo* studies have identified interacting regions in Stat3 and cJun that participate in cooperative transcriptional activation (Zhang *et al., 1*999). Interestingly, transcription of injury related enzyme DINE (damage induced neuronal endopeptidase) increased several-fold when transcription factor Sp1 acts as a scaffolding protein, mediating recruitment of Atf3, c-Jun and Stat3 to the DINE promoter in response to LIF upregulation (Kiryu-Seo et al., 2008). The zebrafish homologue of LIF – M17 is upregulated in response to optic nerve lesion in fish (Elsaeidi *et al*., 2014) and regeneration specific regions of the fugu *gap43* promoter also contain a putative site for Sp1. Future studies may be carried out to test the possibility that Sp1-mediated recruitment of candidate transcription factors is also involved in our CNS injury paradigm (Fig.19).

In conclusion, using *gap43* regulation as a model, we have identified a highly conserved transcription factor network comprised of Ascl1a, Atf3, c-Jun and Stat3 as important mediators of successful CNS regeneration. In the future, more

focus will be placed on understanding how this network of transcription factors regulates other growth-associated genes and molecular components to eventually generate a gene regulatory code driving successful CNS regeneration in fish. Future studies should also focus on evaluating whether the same cohort of transcription factors are also required for other phases of regeneration namely axon guidance and synaptic refinement. Ulitmately, identifying specific regulatory mechanisms shared by several important regeneration genes will allow for optimal development of gene-based therapies.

Evolutionary conservation of regeneration gene regulatory pathways

Previously we have shown that *gap43* promoter regions involved in regulating developmental axon growth are distinct from regions required for regenerative axon growth (Kusik *et al*., 2010). Furthermore, we found that *cis-acting* enhancer sequences required for regenerative axon growth are highly conserved across teleosts, but not mammals. These findings implied that these teleost conserved *cis* elements and the transcriptional pathways that impinge on these regeneration specific elements may contribute to species-specific differences in regenerative ability. The work presented here further supports the idea that regulatory mechanisms supporting regeneration-associated gene expression in response to CNS injury are well conserved in teleosts. We find that in addition to the *cis-acting* elements, the *trans-acting* regulatory pathways are also highly conserved between pufferfish and zebrafish, which diverged approximately 300 million years ago (Yamanoue *et al*., 2006). Importantly, our work in conjunction with that of others on Ascl1a/Mash1 suggests that the downstream targets of the

regeneration-associated transcription factors are also conserved between fish and mammals, making transcription factor gene therapy a potentially feasible therapeutic approach. Together these results substantiate the use of fish to identify factors that are essential for successful CNS regeneration followed by subsequent validation in mice, with the ultimate goal of developing therapeutic approaches to promote regeneration in human patients suffering from CNS injuries or diseases.

Although our results show that Ascl1a, Atf3, c-Jun, and Stat3 all contribute to the activation of *gap43* expression in regenerating neurons, we cannot conclusively distinguish whether the effect is due to direct binding of the transcription factors to the *gap43* gene or indirect regulation through other transcription factors. Chromatin immunoprecipitation (ChIP) assays on regenerating retina, will help determine whether our candidate transcription factors bind to *fugu* promoter regions *in vivo* to drive regenerative *gap43* expression. If candidates do bind endogenous *fugu* promoter regions, specific *cis-acting* sequences bound to transcription factors *in vivo* will be delineated.

The next step is to determine if binding sites for these critical transcription factors exist within the mammalian *gap43* promoter/enhancer. Udvadia *et al*., 2001 demonstrated that a 1kb regulatory region from rat *gap43* promoter was able to direct transgene expression during nervous system development in zebrafish; however, the same rat *gap43* promoter fragment was insufficient to direct

expression of transgene during regenerative growth. Furthermore, regeneration-specific *gap43* enhancer sequences that are highly conserved across fish species, but not between fish and mammals (Kusik et al., 2010) suggests that the sequences may have been lost due to genomic rearrangement or mutation. However, functional AP-1 sites and E-boxes, which bind cJun/Atf3 and Ascl1, respectively have been identified with mammalian *gap43* promoters. Thus the lack of sufficient induction of the transcription factors in response to CNS injury, or the lack of the appropriate stoichiometry of the transcription factors is the more likely cause of differences between fish in mammals in the ability to induce regenerative *gap43* expression. Finally, another possible difference in the ability to induce regenerative *gap43* expression between fish and mammals may lie in the accessibility of the transcription factors to promoters of regeneration-associated genes, which is discussed in more detail in the next section.

Epigenetic control of axon regeneration

Understanding epigenetic regulation of axon regeneration has gained wide interest in recent times, with studies uncovering the importance of modifying epigenetic regulators to promote axon regeneration (Puttangunta *et al., 2*014, Finelli *et al*., 2013, Trakhtenberg *et al*., 2012, Maki and Kimura., 2012., Gaub *et al*., 2011). It is being recognized that ultimately the capacity for regeneration might depend on plasticity of the cellular epigenome, which dictates the ability of the cell to respond to injury signals (Barrero *et al*., 2011). For transcription

factors to effectively transcribe their target genes, chromatin remodelers or epigenetic factors are required to provide accessibility to DNA regions. This is perhaps the reason underlying the modest effect on axon re-growth upon Mash1 overexpression in comparison to the robust effects observed in fish. Hence co-expression with molecules/pathways that relax the chromatin such as p300 (Gaub et al., 2011) may be required to form transcriptional complexes that are more capable of inducing changes in gene expression.

The binding affinity for histones to DNA regions far exceeds the binding affinity of transcription factors to DNA regulatory elements. In this context, simultaneous binding of multiple transcription factors like Ascl1a, Atf3 and cJun to the fugu *gap43* promoter likely contributes to maintaining the "openness" of chromatin thereby allowing for increased transcription of *gap43* gene as opposed to a single transcription factor binding event to the promoter (Fig.19). A similar mechanism was observed with engineered synthetic transcription factors and human gene activation (Pablo-Pinera *et al*., 2013). Many of the engineered transcription factors contributed to synergistic gene activation when delivered in combination, even though they failed to activate target genes when delivered alone (Pablo-Pinera *et al*., 2013). Notably, not all combinations of engineered TFs lead to gene activation; certain combinations of synthetic factors caused repression of reporter gene as well. Thus, a thorough understanding of the interactions and epistasis of regeneration-associated transcription factors, and their impact on the chromatin landscape surrounding regeneration-associated genes encoding structural elements of the growth is needed.

Epigenetic modifications in response to axonal injury may provide key clues in understanding how gene expression for select regeneration-associated genes is modulated and sustained in response to injury. Consistent with this, Puttagunta *et al*., 2014 have identified that in response to retrograde signaling following sciatic nerve axotomy, positive chromatin remodeling occurs on the promoters of crucial regeneration associated genes such as *gap43*, *galn* and *bdnf*. Specifically, p300/CBP-associated factor (Pcaf) was responsible for acetylation of lysine 9 on histone H3 associated with promoters of regeneration-associated genes, along with a reduction in methylation of histone 3 at lysine 9. Notably, similar positive chromatin remodeling events are not observed in response to CNS injury. However, forced overexpression of PCAF in primary cultures of cerebellar granule neurons shifted the reduced acetylation on histone 3 of key gene promoters to positive enrichment (Puttagunta *et al*., 2014). These findings are encouraging and support the notion that modifying epigenetic regulators may be one way to stimulate a transcriptional program for key regeneration associated genes in response to injury. Similar chromatin immunoprecipitation assays to decode the epigenetic environment surrounding fugu *gap43* promoter in response to optic nerve injury in fish will clarify whether epigenetic regulatory pathways driving regeneration are conserved across species.

Final summary and future directions

We have identified gene regulatory pathways underlying successful CNS regeneration in fish. We show that *gap43* re-expression post-injury is critical for RGCs axons to re-establish tectal connections following optic nerve transection. Using transcriptional regulation of *gap43* as a model, we have identified a transcription factor cohort composed of Ascl1a, Atf3 and cJun, which is required for driving CNS regeneration in fish. This combinatorial regulatory pathway driving successful regeneration is highly conserved. This research adds to the body of work in understanding cell-intrinsic mechanisms underlying successful CNS regeneration, in an effort to discover novel targets for therapeutic intervention to improve CNS regeneration in mammals.

While we have used *gap43* as a model to identify pathways underlying successful regeneration, our ultimate goal is to identify a gene regulatory network code to target for therapeutic intervention in mammals. To this end, we have initiated efforts in identifying downstream functional gene targets for transcription factor cJun during optic nerve regeneration in fish. cJun was found to be the only transcription factor common between four different expression profiling datasets of PNS injury induced genes, clearly highlighting a role for c-Jun in mediating successful regeneration (Blackmore *et al*., 2012). We have utilized a combination of ChIP-Seq and RNA-Seq strategy to identify genes that are bound *in vivo* to cJun during optic nerve regeneration that also show reduced transcript levels

upon cJun knockdown as confirmed by RNA-seq. Analysis of these datasets will reveal functional targets of c-Jun during optic nerve regeneration and allow us to start building a gene regulatory network that underlies successful regeneration in fish.

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Curriculum Vitae

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Education and Training

Institution	Degree	Year	Major Area
BIT, Sathy, TN- India	BS	2009	Biotechnology
UW – Milwaukee	PhD*	In progress	Neuroscience

*Project – Gene regulatory mechanisms driving central nervous system regeneration in zebrafish

Doctoral advisor – Dr. Ava. J. Udvadia

Positions and employment

Year	Position	
2007	Research intern	Sugarcane Breeding institute Chennai, TN – India
2008	Research intern	Institute for Alternative Medicine Bangalore, Karnataka – India
2009	Research intern	Indian Institute of Technology Chennai, TN – India
2009 – till date	Teaching assistant	Anatomy and Physiology labs UW – Milwaukee
2010 – till date	TA coordinator	Anatomy and Physiology labs UW – Milwaukee

Publications

Published abstracts

Ishwariya Venkatesh, Robert Teal, Ava.J.Udvadia, 2014. Identification of transcription factors necessary for re-establishment of retinotectal projections after optic nerve transection in zebrafish. Society for Neuroscience. Washington DC.

Ishwariya Venkatesh, Robert Teal, Ava.J.Udvadia, 2014. Identification of transcription factors regulating *gap43* gene expression and optic nerve regeneration in zebrafish. International zebrafish genetics and development. Madison,WI

Ishwariya Venkatesh, Ava.J.Udvadia, 2013. Identification of transcription factors regulating *gap43* gene expression during optic nerve regeneration in zebrafish. 6th Aquatic Animal Models for Human Disease & Midwest zebrafish conference. Milwaukee,WI

Ishwariya Venkatesh, Ava.J.Udvadia, 2013. Identification of transcription factors regulating *gap43* gene expression during optic nerve regeneration in zebrafish. Society for Neuroscience. San Diego,CA

Ishwariya Venkatesh, Ava.J.Udvadia, 2013. Identification of transcription factors regulating *gap43* gene expression and axon outgrowth during optic nerve regeneration in zebrafish. International symposium for neuroregeneration. Asilomar, CA.

Ryan Williams*, **Ishwariya Venkatesh**, D.D.Pearse, Ava.J.Udvadia, M.B.Bunge, 2013. Mash1/Ascl1a leads to GAP-43 expression and axon regeneration in the adult CNS. International symposium for neuroregeneration. Asilomar, CA.

Ishwariya Venkatesh, Ava.J.Udvadia, 2012. Identification of transcription factors regulating axon outgrowth and *gap43* expression during optic nerve regeneration in zebrafish. Society for Neuroscience. New Orleans, LA.

Ishwariya Venkatesh, Ava.J.Udvadia, 2012. Probing molecular pathways regulating CNS regeneration in zebrafish. International zebrafish genetics and development. Madison, WI

Ishwariya Venkatesh, Ava.J.Udvadia, 2012.Identification of transcriptional regulatory pathways involved in inducing regenerative *gap43* expression in retinal ganglion cells. Biological Sciences symposium. Milwaukee,WI

Ishwariya Venkatesh, Ava.J.Udvadia, 2011. Promoter analysis of teleost *gap43* elements in regenerating retina. Midwest Regional Zebrafish Conference.Rochester, MN

Ishwariya Venkatesh, Ava.J.Udvadia, 2011. Promoter analysis of teleost *gap43* elements in regenerating retina. Milwaukee Chapter – Society for Neuroscience. Milwaukee – WI

Ishwariya Venkatesh, Ava.J.Udvadia, 2011.Identification of transcriptional regulatory pathways involved in inducing regenerative *gap43* expression in retinal ganglion cells. Biological Sciences symposium. Milwaukee,WI

Ishwariya Venkatesh, Ava.J.Udvadia, 2010. Promoter analysis of teleost *gap43* elements in regenerating retina. Milwaukee Chapter – Society for Neuroscience. Milwaukee – WI

Ishwariya Venkatesh, Ava.J.Udvadia, 2010.Promoter analysis of teleost *gap43* elements in regenerating retina. Biological Sciences symposium. Milwaukee, WI

Honors and Awards

2009	*Summa cum laude* – BS, BIT –India
2009 – 2014	Chancellors award, UWM
2012,2013	Travel awards, Graduate school –UWM
2013	Best poster award Aquatic animal models for human disease/Midwest zfish meeting Milwaukee, WI
2013	Best poster award Collaborative work with Dr.Ryan Williams (Miami Project to cure paralysis) International Symposium for Neuroregeneration, Asilomar, CA

Grant proposals
None

Professional Services
Memberships and Societies

Student member – Society for Neuroscience, 2012, 2013

Volunteer experiences

Chief Science Judge – Lake Sturgeon bowl (2010,2011,2012,2013,2014)

Community outreach – Brain Awareness week (2010,2011,2012)

Research collaborations
2012 - Present
Role of MASH1 transcription factor in driving *gap43* expression during optic nerve regeneration in zebrafish

Project in collaboration with Dr.Ryan Williams (Advisor: Dr. Mary Bunge) - Miami project to cure paralysis, Miami-FL

Manuscripts

R.R.Williams, I.Venkatesh, D.D.Pearse, A.J.Udvadia, M.B. Bunge. MASH1/Ascl1a leads to GAP43 expression and axon regeneration in the adult CNS. (*in revision)*
I.Venkatesh and A.J.Udvadia.

Transcriptional regulatory pathways driving *gap43*
expression and axon outgrowth during optic nerve regeneration in zebrafish (*in preparation)*.