December 2013

The Role of GAP-43 Phosphorylation in Axon Behavior in the Developing Zebrafish Visual System

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THE ROLE OF GAP-43 PHOSPHORYLATION IN AXON BEHAVIOR IN THE DEVELOPING ZEBRAFISH VISUAL SYSTEM

by

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A Dissertation Submitted in

Partial Fulfillment of the

Requirements for the Degree of

Doctor of Philosophy

in Biological Sciences

at

The University of Wisconsin-Milwaukee

December 2013

ABSTRACT
THE ROLE OF GAP-43 PHOSPHORYLATION IN AXON BEHAVIOR IN THE DEVELOPING ZEBRAFISH VISUAL SYSTEM

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Jennifer B. Forecki

The University of Wisconsin-Milwaukee, 2013
Under the Supervision of Ava J. Udvadia, PhD

Developing neurons extend processes to specific targets and establish connections that are essential for future function of the nervous system. One of these processes, the axon, has a motile tip called a growth cone that rearranges its membrane-associated actin cytoskeleton to turn toward or away from environmental guidance cues. Growth associated protein 43 (GAP-43) is one of the most abundant proteins associated with axonal growth cone membranes and is known to modulate the formation and stability of the actin cytoskeleton during axon guidance. Protein kinase C (PKC)-mediated phosphorylation of GAP-43 on serine 42 regulates its interactions with actin. Phosphorylated GAP-43 stabilizes actin filaments in growth cones that are actively pathfinding. Using the developing zebrafish visual system to model axon growth and guidance, we determined the role of GAP-43 phosphorylation during retinal ganglion cell (RGC) axon emergence and growth by disrupting phosphorylation *in vivo*. In zebrafish embryos we induced the expression of a mutant version of GAP-43, GAP43s42, that has a serine to alanine substitution

mutation at position 42. This dominant negative mutant GAP-43 cannot be phosphorylated by PKC. Overexpression of the GAP43s42a mutant early in visual system development gave rise to embryos in which the optic nerve appeared thinner. Further investigation revealed this phenotype was caused by defects in RGC differentiation and axon guidance errors within the retina. Overexpression of GAP43s42a later in development, when RGC axons are actively growing in the optic chiasm, resulted in a loss in the close association of axons in the optic nerve leading to defasciculation and aberrant axon trajectories. We conclude from these results that phosphorylation of GAP-43 plays an important role in regulating RGC differentiation, axon guidance, and axon fasciculation.

To my Mama and Dad

and all those family and friends who have loved me.

All things are possible because of your faith in me.

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LIST OF ABBREVIATIONS

5H-T	5-hydroxytryptamine
ala41	Alanine at position 41
asp41	Aspartic acid at position 41
BrdU	Bromodeoxyuridine or 5-bromo-2'-deoxyuridine
CAMs	Cell adhesion molecules
CDS	Coding DNA sequence
DMSO	Dimethylsulfoxide
DAPI	4’6’-diamidino-2-phenylindole
EDTA	ethylenediaminetetraacetic acid
EK	EKKwill Zebrafish line
f-actin	Filamentous actin
FGF	Fibroblast Growth Factor
FG43	GAP-43 promoter from *Takifugu rubripes*
GAP-43	Growth associated arotein-43
GFP	Green fluorescent protein (EGFP gene)
HPF	Hours post fertilization
HRP	Horseradish peroxidase
HSP	Heatshock promoter
Hsp70	70 kilodalton heatshock protein
IACUC	Institutional animal care and use committee
IRES	Internal ribosomal entry site

kb	Kilobases
NCAM	Neuronal cell adhesion molecule
NGF	Nerve growth factor
oc	Optic chiasm
od	Optic disc
OFL	Optic fiber layer
on	Optic nerve
ot	Optic tract
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline with Tween20
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PKC	Protein kinase C
PTU	1-phenyl-2-thiourea
PVDF	Polyvinylidene fluoride
RGC	Retinal ganglion cell
SDS	Sodium dodecyl sulfate
TBS	Tris buffered saline
TBS-T	Tris buffered saline with Tween20
TNT	Tris-NaCl-Tween buffer

ACKNOWLEDGEMENTS

I would like to express my gratitude to Dr. Ava Udvadia for being my advisor. Allowing me to transfer into your lab changed the course of my life for the better. I can honestly say that no other single person has taught me more about how to be a good scientist. I appreciate all the time and effort you invested in helping me improve. Thank you for everything and I hope someday to provide the same unwavering guidance for my students.

I would also like to thank my committee members: Drs. Jane Witten, Julie Oliver, Chuck Wimpee, and Murry Blackmore. Over the years my committee has provided important feedback that has helped shape my project in many ways. I also had the pleasure of taking many of their courses. I was inspired to become a professor from those courses. I would especially like to thank Dr. Julie Oliver. She was an outstanding professor and I learned so much about teaching a laboratory course from her.

I would like to thank my mother for her unwavering love and support from a very early age. She was always my number one fan. She taught me how to be brave and persevere during tough times. I would not be who I am today, and the scientist I hope to become, without the sacrifices she made to provide for me. From her I also learned that service to others should be a priority in life. A powerful teacher, she instilled a passion for teaching and learning in me.

I would like to thank my father for the countless times you have helped me with a thousands little things. Knowing that I always have you to count on is such a blessing in my life. From you I have leaned the power of kindness and patience with

even the most difficult situations. You have taught me that every student deserves the very best teacher as you were for me. You and my brothers provided unwavering support and faith in my abilities.

I would like to thank all the past and present Udvadia lab members. Working with you in the lab has been a pure joy. The girls have always provided the perfect atmosphere for producing the best science. I am so lucky and so proud to be a part of this group of young talented scientists. I would like to thank Dena Weinberger for being my constant companion in graduate school and for being an excellent example to follow. I would also like to thank Maria Replogle and Ishwariya Venkatesh for always helping me and being outstanding graduate students. I would like to thank the members of Dr Kurt Suvboda labs, especially Surangi Perera and Sreevidya Sambukumar for all you help and support. I would also like to thank Brandon Kusik and Angie Schmoldt for teaching me how to run a lab. Thank you everyone, particularly for believing in me, even when my faith in myself is low. Working with everyone will always be remembered as one of the best experiences of my life.

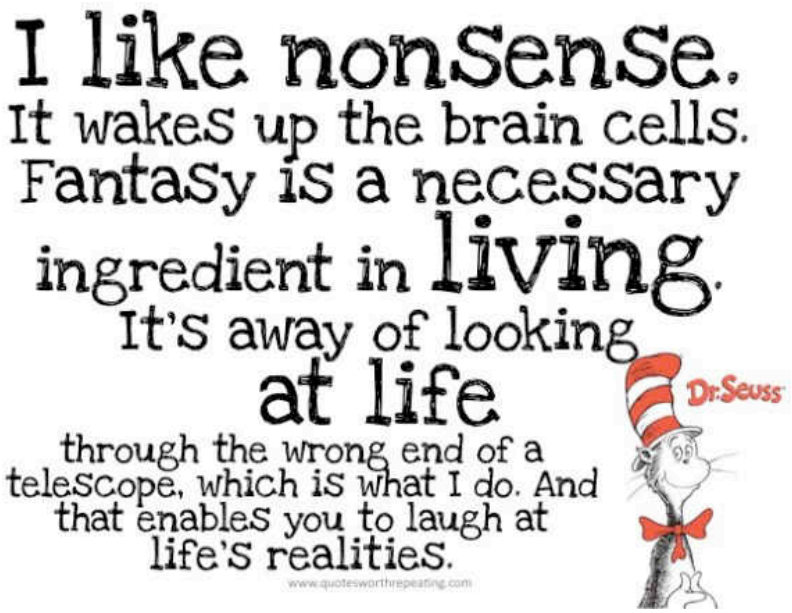
I would like to thank my friends for always supporting me and for the countless hours you have spent listening to me talk about science. You guys have always accepted me for who I am. Thank you Kate Moraine for always listening to my problems no matter how small or how large. From you I have learned so many lessons about grace, kindness, and joy. I wouldn't have been able to do this without you being my voice of reason. I would like to thank Billy Hinbern for the care and consideration you never fail to show me. Having you as my friend makes me a happier and saner person. To Michael, thank you for always being interested in my

work and writing no matter how boring it is. To Jon Weimer, Jordan Mortensen, and Sara Stagg, for always being a constant in my life. You have helped me complete this journey in more ways than I can count.

I would also like to thank all of my students from Cell biology Lab. Being a TA helped me realize that teaching is something that I really love. I am so grateful for everything I have learned from being your teacher.

I would also like to thank the University of Wisconsin-Milwaukee and the biological sciences department and the endowments made to each that helped fund my years of work. I would especially like to thank the AOP fellowship and American Indian Student Services.

I would also like to thank all the fish! You guys are awesome and I could not have done this without you.


I like nonsense.
It wakes up the brain cells.
Fantasy is a necessary
ingredient in living.
It's away of looking
at life
through the wrong end of a
telescope, which is what I do. And
that enables you to laugh at
life's realities.
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CHAPTER I

General Introduction

During the formation of the nervous system, neurons are faced with the challenge of finding the correct target cells among many options. Establishing the correct connections during nervous system development directly correlates with nervous system function in the future. Axons, the cellular processes that grow out from neuronal cell bodies, are tipped with specialized structures called growth cones. Growth cones utilize guidance cues to locate the correct targets within the developing embryo. Receptors located on growth cones bind ligands in the growth environment, and growth cones respond by directing growth towards attractive cues or away from repulsive cues. In order to achieve this response, receptor-ligand interactions trigger intracellular signaling pathways that lead to dynamic changes in the structure of the growth cone. One intracellular target of axon guidance signaling pathways is Growth Associated Protein-43 (GAP-43), which is among the most abundant proteins found in axonal growth cones.

Axon connectivity in the brain is established during development, but it also an important issue in the mature nervous system after injury. Researchers have focused on the requirements for axon growth in an effort to understand how the nervous system forms, but also to determine the capacity for regrowth after injury

in the mature nervous system. To determine the prerequisites of axon growth and guidance, Skene and colleagues focused on proteins present during initial elongation and regeneration of axons and discovered a significant increase in the synthesis and expression of GAP-43 (Skene et al. 1986). In their study, protein extracts fractionated from the growth cones membranes of developing neurons contained 12 times the amount of GAP-43 compared to the membranes from the remainder of the neuron. Immunostaining experiments also demonstrated that GAP-43 preferentially localized to the growth cones of cultured dorsal root ganglion (DRG) neurons (Meiri et al. 1986). The abundant expression of GAP-43 in the growth cones of virtually all neurons, along with evidence that GAP-43 expression declines as the nervous system matures, led to the hypothesized role of GAP-43 in axon growth and guidance (Jacobson et al. 1986).

In vitro experiments proved that GAP-43 is expressed in growing axons during development. Cultured neurons were probed with a GAP-43 antibody to determine if GAP-43 expression is temporally associated with axon development. When initially plated on a permissive substrate, rat superior cervical ganglion cells show high GAP-43 immunoreactivity in the cell body, but during axon extension the immunoreactivity migrated to the growth cones of growing axons (Meiri et al. 1988). Re-localization of GAP-43 to growth cones during axon extension supports a role for GAP-43 in axon development. Another indication that GAP-43 might have a significant role in axon growth is demonstrated by the association of GAP-43 with the cytoskeleton fraction of growth cones isolated from cultured cells (Meiri and Gordon-Weeks 1990). In summary GAP-43 is abundantly expressed in axonal

growth cones where it is associated with the cytoskeleton and could be involved in regulating cytoskeleton dynamics during axon growth and guidance.

GAP-43 knockout mice and axonal guidance

While GAP-43 expression is observed in virtually all developing neurons, it appears to be essential for axon growth and guidance in specific subsets of extending axons. Due to the axon growth-associated expression pattern of GAP-43, the knockout mice model was expected to show extensive defects in axonal outgrowth and guidance. However, while 90% of GAP-43 (-/-) knockout mice died within the first 3 weeks of the postnatal period due to behavioral defects in feeding, the appearance of the nervous system was grossly normal (Strittmatter et al. 1995). Even though the majority of axons were able to extend and grow towards synaptic targets, it is possible that defects caused by removing GAP-43 might be more pronounced during early critical periods and then partially repaired or compensated for later in development. Closer examination of specific axonal tracts within the central nervous system of the knockout mice revealed defects, particularly at locations where axons normally cross the midline.

When observed earlier in development axons in GAP-43 (-/-) knockout mice displayed aberrant trajectories while attempting to cross the midline. Normally in the developing mouse visual system, retinal ganglion cell (RGC) axons reaching the midline either continue to grow within the ipsilateral optic nerve or cross the midline to grow within the contralateral optic nerve. Most RGC axons will cross the midline at the optic chiasm and eventually innervate the contralateral lateral geniculate nucleus. At embryonic day 19, axons from RGCs in GAP-43 knockout

mice stalled in the optic chiasm and projected in aberrant directions (Strittmatter et al. 1995, Zhu and Juliene 1999). There are many axons in the developing nervous system that must cross the midline of the brain including axons in the anterior commissure, hippocampal commissure, and corpus callosum. In the brains of GAP-43 (-/-) knockout mice, axons from each of these tracts reached the midline but failed to cross resulting in the absence of the anterior commissure, hippocampal commissure, and corpus callosum (Shen et al. 2002).

In addition to the guidance defects observed in axons that normally cross the midline, defects were also observed in axons that innervate the cortex to create somatosensory maps in GAP-43 (-/-) knockout mice. In mice the sensory map that represents whiskers is called the ordered barrel array. Creating this map requires the guidance and segregation of axons into an ordered pattern representing the spatial arrangement of whiskers on the face of mice. In GAP-43 (-/-) knockout mice, the ordered array was completely disrupted due to segregation errors involving a subset of axons from the thalamic neurons that normally innervate the barrel cortex to create the cortical maps (Maier et al. 1999). The brains of the GAP-43(-/-) knockout mice also showed a decrease in the number of axons from serotonin (5-HT) neurons in the raphe nucleus that innervate the cortex and hippocampus (Donovan et al. 2002). Segregation errors and guidance errors can be the result of a failure to respond to diffusible guidance cues.

Not only do GAP-43 depleted axons fail to terminate at the correct targets, but they fail to remain in designated pathways and fasciculate with other axons. Axon bundles in the nervous system of GAP-43 (-/-) mice knockout appeared wider.

Prior to reaching the midline the commissural axons in the GAP-43 (-/-) knockout mice displayed a wider defasciculated appearance (Shen et al. 2002). Also in the disrupted barrel array of GAP-43 (-/-) knockout mice, thalamocortical afferents innervated the cortex in a wider area due to defasciculation of axons in the deeper layers of the cortex (Maier et al. 1999). Taken together, these results suggest that GAP-43 is necessary for axon fasciculation in the developing nervous system. Fasciculation is essential for the formation of the nervous system, particularly to allow later developing axons to bundle together with earlier extended or pioneer axons.

GAP-43 is involved in neuronal differentiation

Previous work has demonstrated that GAP-43 expression is present prior to axon extension and fasciculation. Neuronal progenitors, the proliferating cells that are the precursors of neurons in the brain, express GAP-43 just prior to differentiation where it appears to be an important determinate of axis orientation during the asymmetrical division of neuronal precursors (Mani et al. 2001). Initially when studying the brains of GAP-43 (-/-) knockout mice, researchers observed a reduction in the volume of the brains (Maier et al. 1999). Disrupting the axis orientation of dividing neuronal precursors could disrupt the final division before differentiation, causing a reduction in the amount mature neurons. Thus, in addition to its role in axon growth and guidance, GAP-43 may also play a role in promoting the differentiation of neuronal precursors.

To focus on the differentiation of individual neuronal precursors and the integration of GAP-43 into signaling pathways that alter the cytoskeleton during

differentiation, the depletion of GAP-43 was studied in cultured neuronal precursors. Depletion of GAP-43 causes disorganization of the cytoskeleton in cultured neuronal precursors prior to differentiation. During neurogenesis, neuronal progenitors undergo asymmetrical cell divisions producing two daughter cells with different cell fates, one of which differentiates into a neuron. The neuronal cell exits the cell cycle, adopts the polarized neuronal morphology, and extends neurites that will become axons and dendrites. Depleting GAP-43 from cultured neuronal precursors disrupts organization of cellular components during asymmetric cell division and halting the precursors before the mature neurons are produced. When GAP-43 was completely depleted from differentiating embryonic carcinoma P19 cells, it reduced the number of differentiated neurons and caused abnormal axonal morphology in the remaining cells (Mani et al. 2000). Further investigation illustrated that in cultured cerebellar granule cells, phosphorylated GAP-43 is required to position the molecules responsible for centrosome and microtubule orientation in developing neurons (Gupta et al., 2010). Disrupting the orientation of centrosomes and microtubule disrupts asymmetrical division by altering the axis of division and could also disorganize the bipolar morphology of developing neurons by disrupting the segregation of organelles. GAP-43 in neuronal precursors could be essential for the organization of the actin cytoskeleton responsible for axis orientation and organelle organization.

GAP-43 overexpression in the adult nervous system

While depletion of GAP-43 from the nervous system occurs at the earliest stages of development, the overexpression of GAP-43 has been more extensively

studied in the mature nervous system. Researchers hypothesized that increasing the expression of GAP-43 ectopically in the mature nervous system should potentiate the effects GAP-43 has on supporting the growth and guidance of axons and possibly return mature neurons to a dynamic developmental state with increased competence for growth. When GAP-43 was overexpressed in the mature nervous system, it potentiated spontaneous sprouting in the neuromuscular junction and the mossy fibers of the hippocampus (Aigner et al. 1995). This overexpression experiment was conducted in mature neurons to try to reinstate a developmental capacity for re-growth. Overexpression in the developing nervous system has been less extensively studied.

GAP-43 depletion disrupts growth cone structure in cultured cells

When GAP-43 was depleted from cultured neurons after differentiation and during axonal extension it resulted in a disruption of growth cone morphology. To study the morphology of growth cones when GAP-43 was missing, Aigner and colleagues depleted GAP-43 from cultured DRG neurons with small antisense oligonucleotides, which lowered the expression of GAP-43 to below detectable levels. In their experiments, dissociated control untreated DRGs from E16 chicks regenerated normal growth cones when cultured on permissive substrate like laminin. Cultured DRGs depleted of GAP-43 had either thinner neurites and smaller growth cones on laminin substrate or a complete lack of neurite outgrowth when plated on poly-L-ornithine (Aigner and Caroni 1993). This shows that not only is GAP-43 important for regulating the cytoskeleton during growth cone formation and neurite outgrowth, but it is also important for the response of neurites to

extracellular signals that direct guidance and outgrowth. To further support this hypothesis, nerve growth factor (NGF) failed to induce normal neurite outgrowth and spreading in GAP-43 depleted growth cones (Aigner and Caroni 1995). Also in these experiments, the growth cones of GAP-43 depleted neurons were missing local concentrations of f-actin and displayed poor adhesion to substrate.

Further investigation also indicates that GAP-43 is required for the neurite extension induced by neuronal cell adhesion molecule (NCAM) binding in cultured cells. In cultured P19 cells, the presence of GAP-43 is required for the binding of NCAM-180 to cause modulation of the actin cytoskeleton (Korshunova et al. 2007). This study also showed that phosphorylation of GAP-43 by protein kinase C (PKC) is important for NCAM-induced outgrowth and that GAP-43 and NCAM-180 form a complex (Kroshunova et al. 2007, Kroshunova and Mosevitsky 2010). Thus, in cultured cells GAP-43 is required for inducing axonal outgrowth, particularly in response to extracellular cues like NCAM.

Removing GAP-43 from cultured cells results in severe defects in differentiation, axon outgrowth, and growth cone structure. In this respect, the axon growth phenotypes associated with GAP-43 disruption in cultured cells appear more severe than those observed in the GAP-43 (-/-) knockout mouse. The GAP-43 (-/-) knockout nervous system is grossly normal while the removal of GAP-43 from cultured cells has severe consequences during axon formation. When interpreting the results from *in vitro* and *in vivo* experiments, it is essential to consider that the knockout experiments reflect the removal of GAP-43 from the complex *in vivo* environment. In contrast, cell culture experiments reflect a more simplistic

controlled model of axon growth and guidance. Differences in environmental factors could contribute to the ability of GAP-43 (-/-) knockout mice axons to grow towards targets while the GAP-43 depleted axons in cultured cell experiments were unable to initiate axon outgrowth. These culture experiments demonstrated that GAP-43 is required during specific controlled culture scenarios, but *in vivo,* compensatory mechanisms allow the developing embryo to overcome these deficits. Cultured cells are functioning in an isolated system and defects could be exaggerated without the compensatory mechanisms normally found *in vivo*. This can be especially pertinent when studying the role GAP-43 has in responding to a single guidance cue or adhesion molecule, which in the functioning nervous system is not the sole determinant of axon growth and guidance.

Regulation of GAP-43 activity by phosphorylation

Growth cones in the developing nervous system respond to guidance cues by rapidly changing morphology, and GAP-43 activity must be similarly regulated in order to support the constantly changing axon behavior. Experiments revealed a post-translational modification that can be added and removed to control the activity of GAP-43. *In vitro* observations showed that GAP-43 was phosphorylated on serine 41 in response to PKC stimulation within the highly conserved N-terminus of the protein (corresponds to serine 42 in zebrafish) (Akers and Routtenberg 1985, Routtenberg 1985, Eichberg et al. 1986).

GAP-43 phosphorylation coincides with changes in growth cone structure and occurs in response to guidance signals. Supporting this role was the observation that GAP-43 phosphorylation occurs in the distal part of the growing axons as they

approach their targets, indicating a spatial and temporal regulation of GAP-43 phosphorylation (Meiri et al. 1991). During axon guidance, stationary growth cones, with the highest filopodial motility, actively probe the environment by rearranging their actin cytoskeleton. In these halted growth cones the concentration of phosphorylated GAP-43 is greatly increased and concentrated along the curved turning edge of filopodia engaged in pathfinding (Dent and Meiri 1992,1998). Phosphorylated GAP-43 is clustered on the edges of membranes that are actively rearranging based on guidance cues, indicating that phosphorylation could be involved in the local actin rearrangement required to turn the growth cone in response to the binding of guidance molecules. Supporting this hypothesis, evidence from previous research shows that PKC phosphorylates GAP-43 in response to target derived neurotrophic molecules like NGF and CAMs (Meiri and Burdick 1991, Meiri et al. 1998).

Both *in vivo* and *in vitro,* a mutation at the GAP-43 phosphorylation site leads to the disruption of actin organization during the development of neurons. Studies demonstrating the role of GAP-43 phosphorylation manipulated the PKC phosphorylation site of GAP-43, at serine 41 either by blocking phosphorylation with an alanine substitution at position 41 (GAP-43 ala41) or by simulating the addition of a phosphate group with an aspartic acid substitution at position 41 (GAP-43 asp41). Expression of the two different mutants has different effects on axons growth and guidance. In non-neuronal cells, expression of GAP-43 leads to extensive spreading and an f-actin radial array supporting numerous filopodia. These effects were completely lost when the cells instead expressed GAP-43 with

serine 41 replaced with an alanine rendering GAP-43 ala41 unphosphorylatable (Widmer and Caroni 1993). In cultured neuronal cells, the expression of GAP-43 ala41 led to irregular blebbing of the membrane and thinner neurites, and disrupted adhesion to a laminin-coated surface (Meiri et al. 1996).

In cultured cells, stable overexpression of the pseudophosphorylated GAP-43 asp41 or the unphosphorylateable GAP-43 ala41 had different effects on the morphology of growth cones. This was illustrated when the GAP-43 asp41 overexpression potentiated filopodia extension, f-actin formation and neurite outgrowth in response to NCAM binding. In contrast GAP-43 ala41 immunoprecipitated with neruotubulin, and did not potentiate NCAM induced outgrowth (Nguyen et al. 2009). These studies indicate that phosphorylated GAP-43 is required *in vitro* to regulate growth cone morphology in response to external signals.

GAP-43's association with the actin cytoskeleton of growth cones was found to be dependent on the phosphorylation state of GAP-43. A final aspect to understanding the role of GAP-43 during axon development is to determine if GAP-43 directly associates with the cytoskeleton and if that association is dependent on the phosphorylation state of GAP-43. In a cell free assay, phosphorylated GAP-43 disrupted phallodin association by binding laterally to actin filaments and stabilizing their structure enough to lower the critical concentration of actin monomers required for polymerization (He et al. 1997). In contrast actin monomers were capped by unphosphorylated GAP-43 and prevented from polymerizing

further (He et al. 1997). Together these studies suggest that phosphorylation is a key regulator of GAP-43 association with actin.

The *in vivo* effects of phosphorylation on GAP-43 activity has been less extensively studied. Yet, some studies indicate that the phosphorylation of GAP-43 *in vivo* is important for the development of the nervous system. Phosphorylation in cultured cells appears to be associated with axon growth/guidance and the differentiation of neuronal precursors (Gupta et al. 2010, Meiri et al. 1998). It is hypothesized that GAP-43 phosphorylation is associated with these activities *in vivo.* Researchers were able to show that in the rat cortex the expression of phosphorylated GAP-43 is associated with cells undergoing horizontal cell divisions as part of cortical neuron differentiation (Stricker et al. 2006). Taken with the previous cell culture data, researchers hypothesize that phosphorylated GAP-43 supports f-actin during the final cell division and axon emergence.

Later in development GAP-43 phosphorylation appears to be important for fasciculation of axons in the nervous system. In mice, when unphosphorylatable GAP-43 ala41 was expressed during development, the mossy fibers of the hippocampus grew ectopically into the distal region, which was caused by defasciculated axons (Holahan et al. 2010). This indicates that phosphorylation is a key regulator of GAP-43 activity during the fasciculation of axons. Phosphorylated GAP-43 could regulate actin assembly in response to NCAM binding and FGF receptor activation which are integral for fasciculation (Masai et al. 2003, Meiri et al. 1998, Thanos et al. 1984). In the visual system of zebrafish, pan-neuronal expression of wild type GAP-43 or pseduphophorylated GAP-43 asp42 promoted

the branching of the terminal arbors of RGC in the tectum. Expression of GAP-43 ala42 using the same promoter instead caused immature arbors with longer and skinnier branches (Leu et al. 2010). The latter study focused on RGC axon innervation of the tectum, yet little is known about the earlier effects of GAP-43 phosphorylation on RGC axon development preceding innervation, specifically axon growth and guidance in the retina and optic chiasm.

Researchers studied the effects of overexpressing GAP-43 mutated at the phosphorylation site in the brains of rats and mice during learning and memory tasks. Mice overexpressing pseudophosphorylated GAP-43 asp41 displayed enhanced spatial and contextual memory retention during memory tasks and this enhancement was lost when GAP-43 ala41 was overexpressed instead (Holahan and Routtenberg 2009, Holahan et al. 2010). Previous results *in vivo* the proved that GAP-43 is required for proper nervous system development and these results demonstrating the importance of phosphorylation being the key regulator of GAP-43 activity has led to the current investigation concerning the developmental role of GAP-43 phosphorylation *in vivo*.

GAP-43 activity demonstrated in the developing visual system

During the development of the visual system, RGC axon formation progresses through several developmental steps that could require GAP-43 to regulate the cytoskeleton, making the visual system the ideal model to study GAP-43 phosphorylation *in vivo*. The RGCs of the retina are the first population of cells to differentiate, starting at 28 hours post fertilization (hpf), after emerging from the asymmetrical cell divisions of the neural progenitors in the retina (Fig. 1).

Regulation of cytoskeletal elements, by proteins like GAP-43, during cell cycle exit is essential for cell polarity and the proper organization of organelles. Experiments discussed previously demonstrated that GAP-43 is involved in cellular division during neuron differentiation. After differentiating, RGCs extend neurites and one of these will develop into an axon. Experiments looking specifically at the zebrafish visual system, demonstrated that exposure to guidance molecules in the basal lamina is required for RGC polarization and axon extension (Zolessi et al. 2006). In isolated growth cones, GAP-43 was required to bind to laminin substrates, a common component of the basal lamina (Meiri et al. 1996).

Communication between cellular components like the cytoskeleton and signaling molecules is another way to establish the polarity of cells in the relation to the external environment. GAP-43 is phosphorylated and supports the actin cytoskeleton in response to extracellular signals like fibroblast growth factors (FGFs) and NCAMs both of which are important for the designation of neuron polarity and axon extension during differentiation (Maier et al. 1999, Mani et al. 2001, Meiri et al. 2001).

Axons emerge from newly differentiated RGCs and starting at 30 hpf growth cones located on the distal tips of these axons navigate the layers of the retina to exit the eye into the optic nerve (Fig. 1). Phosphorylated GAP-43 is expressed in the growth cones of RGCs and is responsible for organizing the actin cytoskeleton in response to guidance cues. In zebrafish and other animal models the ability of

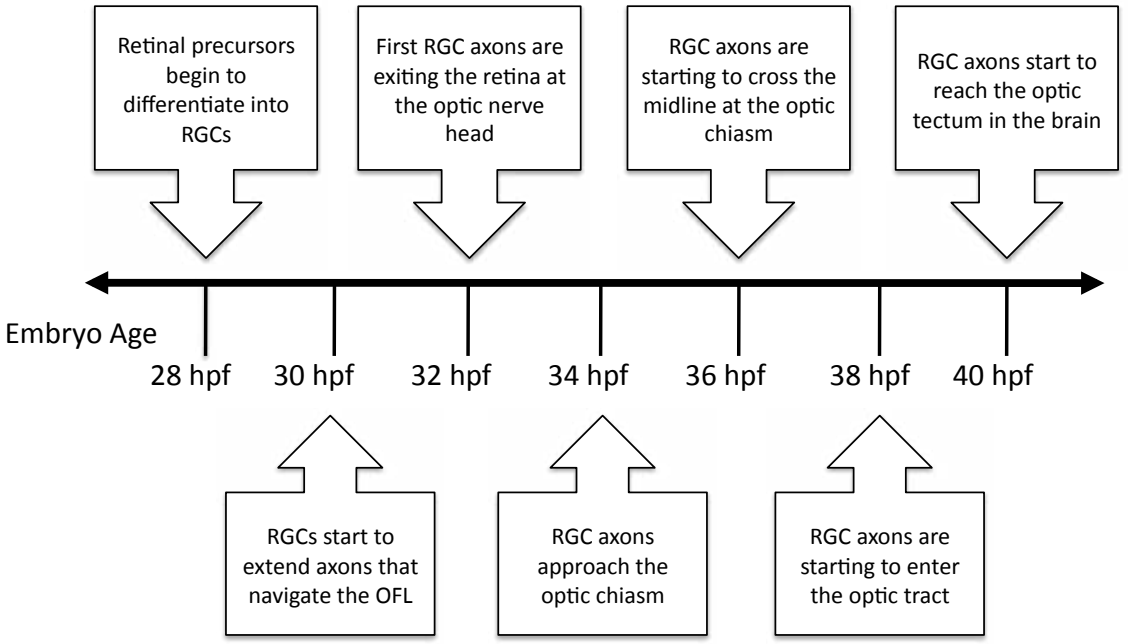

Figure 1. Timeline of RGC axon development in the zebrafish retina. This diagram is a timeline of RGC development starting from RGC differentiation at 28 hpf. The timeline continues till the RGC axons have completely crossed the optic chiasm and reach the tectum. It is important to note that the times represented in the timeline are the age of the embryo at the initiation of the events. Due to the wave like progression of development, populations of retinal cells progress through the developmental stages in a staggered fashion.

growth cones to respond to guidance cues is important for guidance within the layers of the retina (Huston et al. 2003, Thompson et al. 2006). Fasciculation is also a key guidance mechanism of RGC axons through the retina; adhesion molecules like neurolin, NCAM, NrCAM and L1 have all been implicated in the fasciculation and exit of RGC axons at the optic disc (Ott et al.1998, Zelina et al. 2005, Bastmeyer et al. 1995, Meiri et al. 1998). NCAM depletion from RGCs results in the failure of RGC axons to exit the retina and removal of GAP-43 phosphorylation in the RGCs could replicate these results (Zelina et al. 2005).

As RGC axon progress across the midline it requires the cytoskeleton of the growth cones to respond to guidance cues in the optic chiasm (Fig 1). *In vivo* GAP-43 has previously been shown to be essential for the guidance of RGCs axons. RGC axons in GAP-43(-/-) knockout mice showed severe pathfinding errors at the midline of the optic chiasm (Zhu et al. 1999, Strittmatter et al. 1995). Other studies show that the axons of cultured RGCs required GAP-43 to respond to guidance signals from the lateral diencephalon cells (Zhang et al. 2013). The visual system therefore offers the opportunity to study the role of GAP-43 phosphorylation in the growth and guidance of axons.

Thesis statement

GAP-43 has an established role in regulation of the cytoskeleton of growing axons *in vitro* (Meiri and Gordon-Weeks 1990). Moreover, it has been demonstrated that phosphorylation by PKC regulates GAP-43 by inducing changes in its ability to interact with and stabilize f-actin (He et al. 1997, Shen et al. 2002, Nguyen et al. 2009). In cell culture experiments the PKC phosphorylation site appears to be

essential for GAP-43 to direct the organization of the neuronal cytoskeleton in response to external signals (Korshunova et al. 2007, Widmer and Caroni 1993). The goal of our experiments is to determine the *in vivo* significance of the evidence regarding phosphorylation-dependent regulation of the cytoskeleton by GAP-43. We have focused on the developing visual system in zebrafish to assess the role of GAP-43 phosphorylation on the guidance of RGC axons through the retina, the optic chiasm, and into the optic tract. At the outset of these studies, we hypothesized that the regulation of GAP-43 by phosphorylation is required for the proper growth and guidance of RGC axons *in vivo*. Our approach to testing this hypothesis was to visualize how overexpression of a phosphorylation deficient form of GAP-43 affects growing axons from newly differentiated RGCs. We expected that by disrupting the phosphorylation of GAP-43, we would uncouple downstream changes in the actin cytoskeleton from external guidance signals that activate PKC. We predicted that the effect of disrupting these pathways would result in the aberrant behavior of axons in the developing visual system.

CHAPTER II

Material and Methods

Maintenance of fish

All experimental procedures involving zebrafish and the maintenance of zebrafish populations were approved by the Institutional Animal Care and Use Committee (IACUC). Zebrafish colonies were maintained as previously described (Westerfield, 2000, Hammond 2010). Adult fish were housed in recirculating rack systems (Aquatic Habitats, Apopka, FL) at 28.5°C on a 14 hour light, 10 hour dark cycle. Fish were fed twice daily with Artemia and/or Zeigler Adult Zebrafish Complete Diet (VWR, West Chester, PA). Fertilized eggs were generated by natural spawning in tanks with a mesh bottom that allowed eggs to remain separated from adults prior to collection. Embryos and fry were kept in 30% Danieau embryo media (17.4 mM NaCl 0.21 mM KCl, 0.12 mM $MgSO_4$, 0.18 mM $Ca(NO_3)_2$) which was changed daily. Fry were raised in an incubator under the same temperature and light conditions as the adults, in static tanks with daily water changes for two-three weeks, and fed with GP Larval Diet (50 - 100 μm and 100 - 200 μm; Brine Shrimp Direct, Ogden, UT) and Artemia. Fish and embryos from the EKKWill strain (EkkWill Waterlife, Ruskin, FL) were used for transient microinjection studies, preparation of lysates for western blots and generation of transgenic lines. Embryos from the

Tg(Frgap43:EGFP)mil1 strain (Udvadia, 2008) were used for transient microinjection and visual system studies. Embryos from the HSP-GAP43s42a-IRES-nucGFP, HSP-GAPWT-IRES-GFP, and HSP-nucmCherry-IRES-GFPCAXX lines (established as part of the studies described herein) were used for stable transgenic visual system studies, retinal cell counts, and western blot procedures.

Generation of a point mutation in PKC phosphorylation site of GAP-43

To generate an unphosphorylatable GAP-43 protein, we induced a dinucleotide substitution mutation (AG to GC) in the *gap43* gene, which resulted in an amino acid substitution of serine 42 to an alanine (GAP43s42a). The substitution mutation was created using the Phusion Site-Directed Mutagenesis kit with high-fidelity phusion hot start DNA polymerase and the following primers for the zebrafish *gap-43* CDS: forward primer AAGATCCAGGCCGCCTTCCGCGGACAC (mutated codon is underlined), and reverse primer TGGTGGCCGCC TTGTGAGCGTTTTCCTCC (Thermo Scientific. Pittsburgh, PA). PCR was carried out with standard reagents and procedures. In zebrafish, introducing this mutation in the protein kinase C phosphorylation site makes the protein product unphosphorylatable (Leu et al. 2010). The GAP43s42a mutant amplicon was ligated into the pGEM-easy vector (Invitrogen, Grand Island, NY) and sequenced to confirm the mutation.

Construction of GAP-43 transposon expression vectors

In order to develop transient and stable transgenic zebrafish expressing mutant GAP-43, we first created transposon expression vectors. To generate our

transposon constructs, we made use of existing plasmid components in the Tol2kit generously made available to the zebrafish research community by the laboratory of Dr. Chi-Bin Chien (Kwan et al. 2007). The Tol2kit consists of a series of modular components (promoters, coding sequences, tags) that can be recombined into a destination vector containing the tol2 transposon backbone using Gateway Cloning (Invitrogen, Grand Island, NY). We used a combination of existing entry clones and new entry clones, which are listed in Table 1 and will be described in further detail below.

The 5' prime elements contained promoter sequences used to express the GAP-43 cDNA. For these experiments we used two different 5' prime element vectors with either the *hsp70* promoter (HSP) or the fugu GAP-43 promoter (fG43). The p5E-hsp70 vector contains the *hsp70* heat inducible promoter. The heatshock promoter is a 1.5 kb fragment that is located directly upstream of the *hsp70* gene (Halloran et al. 2000). The promoter contains arrays of heatshock elements that activate transcription when heated to 38° C (Shoji and Sato-Maeda 2008). fG43 is the promoter for the *Takifugu rubripes* (fugu) *gap43* gene. fG43 was sub-cloned and ligated into the 5' prime vector with a multiple cloning site (p5E-MCS). The isolation and production of the 3.6-kb fragment of the fugu *gap43* promoter has been previously described (Udvadia 2008).

The middle elements contained cDNA encoding mutant (GAP43s42a) or wild type (GAP43WT) GAP-43. Gene specific primers were used to add BP recombinase attB site sequences to 5' and 3' end of the GAP43s42a and GAP43WT cDNA sequence (Forward BI site GGGGACTGCTTTTTTGTACAAACTTGGTCG

Table 1. Gateway cloning vectors	
Name	Description
1. 5' prime element entry vectors	
p5E-hsp70	*hsp70* promoter (Tol2 kit)
p5E- fG43	GAP-43 promoter from *Takifugu rubripes*
2. Middle element entry vectors	
pME-GAP43s42a-myc	GAP43s42a gene with myc tag
pME-GAP43WT-myc	GAP43WT gene with myc tag
pME-nucmCherry	nuclear-localized mCherry gene (Tol2 kit)
3. 3' prime element vectors	
p3E-IRES-GFP	IRES driven GFP gene (Tol2 kit)
p3-IRES-nucGFP	IRES driven nuclear directed GFP gene (Tol2 kit)
p3E-IRES-GFPCAXX	IRES driven membrane directed GFP gene (Tol2 kit)
4. Destination vectors	
pDESTol2pA2	Gateway cloning vector flanked by Tol2 inverted repeats (Tol2 kit)

Table 1. Gateway cloning vectors. The table lists the gateway cloning vectors that were used to generate expression vectors. A 5' prime element, middle element, and 3' prime element from groups 1, 2, and 3 were recombined together into the destination vector pDESTol2pA2. The vectors are part of the Tol2 kit are indicated and the others were generated in this study using tools from the Tol2 kit and sequences cloned from zebrafish and *fugu* GAP-43 gene loci.

AGAGGCCTTGAATTCAA, Reverse B2 site GGGGACCACTTTGTACAAGAAAGCT GGGTCCAACTTTGTA TAATAAAGTT). Primers were also used to add a myc epitope tag to the GAP43WT and GAP43s42 cDNA sequences (Forward GGGGACTGCTTTTT TGTACAACTTGGTCGAGAGGCCTTGAATTCAA, Reverse GGGACCACTTTG TACAAGAAAGCTGGGTGACCAAAAGCTCATTTCTGAAGAGGACTTGA). PCR was carried out with standard reagents and procedures. The enzyme BP recombinase (Invitrogen) recombined the PCR products flanked with attB sites, with a donor vector (middle entry donor vector pDONR221) containing attP sites. The result is middle entry vectors containing GAP43s42a and GAP43WT (pME-GAP43). As a

control, the middle entry vector encoding nuclear-targeted mCherry fluorescent protein was also used a middle element entry vector (pME-nucmCherry).

The 3' elements contained cDNAs encoding cytosolic EGFP or nuclear-targeted EGFP preceded by an internal ribosomal entry site (IRES) from encephalomyocarditis virus and the poly A tail (p3E-IRES-GFP). For the remainder of these experiments EGFP will be referred to as GFP. This element was combined with the previous element to create a bicistronic mRNA. A control expression vector was generated using the 3' prime vector containing the cDNA for membrane-targeted GFP (p3E-IRES-GFPCAXX).

An LR recombinase reaction was carried out according to the protocol outlined in the Tol2kit. The reaction used the LR recombinase and attR sites in the 5' prime, middle, and 3' prime entry vectors to recombine with attL sites in the destination vector pDESTol2pA2. Because each entry element utilizes unique recombination sites, the 5' prime, middle, and 3' prime elements become aligned in the proper order. Table 2 shows the HSP-GAP43s42a-IRES-nucGFP, HSP-GAPWT-IRES-GFP, HSP-nucmCherry-IRES-GFPCAXX, fG43-GAP43s42a-IRES-GFP, and fG43-GAP43WT-IRES-nucGFP expression vectors.

Transient transposon transgenesis

Expression vectors were injected into the yolk-free cytoplasm of 1-cell staged embryos from the EK or Tg(Frgap43:EGFP)mil1 strain to create stable or transient transgenic zebrafish respectively. To induce genome insertion, the vectors were coinjected with Tol2 transposase mRNA as previously described (Suster et al. 2009).

Table 2. Tol2 expression vectors	
Name	Description
HSP-GAP43s42a-IRES-nucGFP	Heatshock promoted GAP43s42a and IRES driven nuclear directed GFP
HSP-GAP43WT-IRES-GFP	Heatshock promoted GAP43WT and IRES driven GFP
HSP-nucmCherry-IRES-GFPCAXX	Heatshock promoted nuclear directed cherry and IRES driven membrane directed GFP
fG43-GAP43s42a-IRES-nucGFP	*Fugu* GAP-43 promoted GAP43s42a and IRES driven nuclear directed GFP
fG43-GAP43WT-IRES-GFP	*Fugu* GAP-43 promoted GAP43WT and IRES driven GFP

Table 2. Tol2 expression vectors. Expression vectors were generated by LR recombinase reaction using the elements from table 1. HSP-GAP43s42a-IRES-nucGFP, HSP-GAP43WT-IRES-GFP, and HSP-nucmCherry-IRES-GFPCAXX vectors use the HSP promoter to drive expression of the transgene using heat as an induction method. fG43-GAP43s42a-IRES-nucGFP, and fG43-GAP43WT-IRES-GFP use the *fugu* GAP-43 promoter to drive expression of the transgenes in a pattern that mimics endogenous GAP-43. Each expression vector also contains the gene for GFP preceded by an IRES element. The expression vectors are bicistronic because of the addition of the secondary ribosomal entry site.

The capped transposase mRNA was produced using the mMessage Machine SP6 kit (Ambion Invitrogen) according to manufacturer's directions, using the pCS2FA-transposase plasmid (gift of Dr. Koichi Kawakami) as a template. Transposase mRNA was treated with Turbo DNase (Ambion), extracted with phenol/chloroform and stored in nuclease-free water at -80°C. Injected embryos were allowed to develop at 28.5°C in 30% Danieau buffer supplemented with 3 mg/ml 1-phenyl-2-thiourea (PTU) at 8 hours to block pigmentation.

Heatshock of embryos

Transgene expression in embryos injected with HSP-driven expression vectors was induced by heatshock. Embryos were raised to the desired stage at

28.5°C and then transferred to a scintillation vial in 30% Danieau. The vial was heated for 1 hour at 38°C in a re-circulating water bath to induce transgene expression. The vial was then returned to 28.5°C and after 30 minutes the 30% Danieau was replaced with fresh solution. To perform the double heatshock, the same procedure was carried out twice to the same clutch of embryos. Two hours after heatshock, embryos that were injected with or had inherited the HSP-GAP43s42a-IRES-nucGFP or HSP-GAP43WT-IRES-GFP transgenes were screened for GFP expression in the retina. Those with retinal expression were kept for future imaging. Embryos were then either imaged live or preserved in 4 % paraformaldehyde (PFA) overnight at 4°C.

Breeding and screening stable transgenic strains

Embryos from the Ekkwill (EK) wild type zebrafish lines were injected with expression plasmids according to the procedure above. For future breeding, the injected embryos were raised to adulthood under the conditions outlined in the zebrafish maintenance section. When the injected embryos reached 3 to 6 weeks of age they were segregated into breeding pairs with uninjected Ek fish of equal size or age. After the breeding pair successfully laid a clutch, it was raised to 24hpf and heated for 1 hour at 38°C to induce expression of the transgene. Fish that had integrated the transgene into the genome of a population of their germ cells were identified based on the expression of systemic GFP in their progeny, which is part of the bicistronic mRNA encoded by the transgenes. Fish that produced GFP positive progeny were designated founder fish (F0) and were used to establish the next

Table 3. Independently segregating lines for each HSP construct
HSP-GAP43s42a-IRES-nucGFP lines
1. XA HSP-GAP43s42a-IRES-nucGFP
2. XH HSP-GAP43s42a-IRES-nucGFP
HSP-GAP43WT-IRES-GFP lines
1. XD HSP-GAP43WT-IRES-GFP
2. XC HSP-GAP43WT-IRES-GFP
HSP-nucmCherry-IRES-GFPCAXX
1. XE HSP-nucmCherry-IRES-GFPCAXX
2. XB HPS-nucmCherry-IRES-GFPCAXX

Table 3. Independent segregated lines for each HSP construct. Injected zebrafish with germ cell genome insertion were selected as founder fish or F0 generation. For each construct two founder fish were selected and bred with wild type EK zebrafish to create two independently segregating transgenic lines. Lines were named for their construct and the letter assigned to the founder that generated the line. For example, HSP-GAP43s42-IRES-nucGFP fish A was the founder for XA HSP-GAP43s42a-IRES-nucGFP line.

generation. Progeny from an outcross of the founders were heatshocked, and those with GFP expression were raised to sexually maturity to create the F1 generation. Groups of ≈20 F1 generation fish were bred together and progeny from the group mating were raised to sexual maturity to generate the F2 generation. To determine if F2 individuals were homozygous or heterozygous for the transgene, they were outcrossed to fish from the EK strain and progeny were screened for GFP expression after heat induction as described above. Homozygous fish produced 100% GFP-positive progeny, while heterozygous fish produced 50% GFP-positive fish. Homozygous adult fish were kept for producing embryos for experimentation and to establish future generation of the transgenic lines. Two independently

segregating lines were established for each of the constructs (Table 3). Each line is named after the original breeding pair and the letter assigned to the founder fish.

Cell proliferation assay

After embryos were induced to express GAP43WTor GAP43s42a by heatshock at either 28 hpf or 30 hpf, the embryos were later exposed to BrdU (5-bromo-2'-deoxyuridine) to label dividing cells. At 47 hpf, embryos were incubated with 10mM BrdU (Sigma-Aldrich, St. Louis, MO) in 30% Danieau with 15% dimethylsulfoxide (DMSO) for 30 minutes on ice. After the incubation, embryos were rinsed 3 times with 30% Danieau and then returned to 28.5°C. Embryos were preserved in 4% PFA at 48 hpf. After being rinsed in phosphate-buffered saline (PBS) (137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 2mM KH_2PO_4) embryos were prepared for sectioning.

Sectioning Embryos

Preserved embryos were cryoprotected in 20% sucrose in PBS and then embedded in flexible plastic molds in embedding medium (Histoprep and 20% sucrose solution at of ratio of 2:1, VWR, Randar, PA) and frozen on dry ice or at -80°C. Transverse sections (20μm) were made through the heads of the embryos using a Histostate Micotome cryostat (Reichert, Munich, Germany) and mounted on Superfrost plus slides (VWR).

Immunohistochemistry

Immunohistochemistry was carried out on sectioned tissue or whole embryos. To label the axons, whole embryos were stained with zebrafish specific mouse mono-colonal Zn-5 antibody ((1:500)Developmental Studies Hybridoma

Bank, Iowa city), a pan-neuronal antibody that labels the membranes of most axons in the zebrafish nervous system. In retinal sections we used anit-BrdU antibody (Sigma-Aldrich) to probe for cells with proliferative capabilities that have incorporated BrdU into the nucleus. The retinal sections were also probed with anti-active caspase III (R&D System, Minneapolis, MN) to label cells in the retina that were undergoing apoptosis.

Sectioned embryos were washed and hydrated with Tris-NaCl-Tween (TNT) buffer (10mM Tris-HCl, pH 8.0, 150mM NaCl and 0.05% Tween-20). Embryos were then incubated in blocking solution (10% normal calf serum in TNT) for 1 hour and then incubated with primary antibodies diluted into TNT buffer overnight at 4°C. The following primary antibodies were used in this study: anti-BrdU (1:1000), and anti-active caspase III (1:1000). After the primary antibody, sections were rinsed 10 times with TNT buffer. For these experiments we used goat anti-mouse secondary antibody conjugated to either the Alexafluor 546 or 488 (1:1000) (Molecular Probes Invitrogen) diluted in TNT buffer and incubated at room temperature for 2 hours. After incubation in the secondary antibody, embryos were washed 10 times in TNT buffer. Slides were then coverslipped immediately in Vectashield hard-set mounting medium with DAPI counterstain (4'6'-diamidino-2-phenylindole; Vector Laboratories, Burlingame, CA).

Immunohistochemistry was conducted on whole embryos preserved in 4% PFA overnight at 4°C. Whole embryos were treated with acetone and digested with collagenase (0.001g/ml) prior to washing with PBS-T (137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 2mM KH_2PO_4, 0.05% Tween-20). Embryos were incubated for 1

hour in block (PBS, 1% Triton-100, 10% normal calf serum) at room temperature. Whole embryos were incubated with the primary antibody Zn-5 (1:1000) diluted in blocking solution and incubated overnight at 4°C similar to the procedures with sections. After being washed 3 times for 30 minutes each in PBS-T, the embryos were incubated with the goat anti-mouse secondary antibody conjugated to Alexafluor 546 (Molecular Probes Invitrogen) (1:1000) and diluted into PBS-T. Embryos were washed 1 time with PBS-T and sunk into 80% glycerol for future imaging.

Flat mounted retina preparation

Immunostained embryos were transferred into increasing concentrations of glycerol to a final concentration of 80% glycerol. While embryos were held in place with a forceps, an insect pin was used to peal back the cornea and dislodge the lens from the eye of the embryo. Alternatively, the embryo was placed in a dish with 1% agarose and the retina was rubbed along the agarose to remove the cornea and dislodge the lens. The retina was then removed from the embryo using an insect pin to disconnect it from the periorbital space and sever the optic nerve. A small slit was made to flatten the retina, which was mounted in 80% glycerol between two coverslips for imaging.

Measurements of the optic nerve

Embryos that were fixed and labeled with Zn-5 were mounted in glycerol and imaged from the ventral aspect. Once the images were compiled and the dimensions calibrated in Metamorph®, the optic nerve was measured. The diameter of the optic nerve was measured directly after it exited the retina, or at a distance of 75 μm after

the nerves transected at the optic chiasm. Graphical representation of the data show the average diameter at each age analyzed. One-way ANOVA was performed using the diameters of the different treatments as independent sample sets to determine if the differences in diameter were significant.

Fluorescent microscopy

Live embryos were anesthetized in Tricaine MS-222 (1 mg/ml in 30% Daneau) and embedded in 0.5% agarose to immobilize them for image capture using an inverted fluorescence microscope (Nikon TE2000) equipped with a cooled CCD camera (CoolSNAP ES, Photometrics,Tucson, AZ) and Metamorph® imaging software(Molecular Devices, Sunnyvale, CA). Fixed embryos were cleared in 80% glycerol and imaged with the same microscopes. Live and fixed embryos were also imaged with an inverted confocal microscope (Nikon Eclipse TI) using the EZ-C1 program (Nikon). Image processing and quantitative measurements were performed using Metamorph® imaging software after each image was calibrated for pixel size.

Retinal cell counts

Metamorph® software was used to count the number of positively stained cells in images captured from the Zn-5, BrdU, or active caspase 3 immunostaining experiments described above. For each retina, the section containing the optic nerve was used for quantifying the number of cells labeled with each antibody. To select a subset of the retinal cells to count, a rectangle that measured 300 μm by 100μm was overlaid on the image with the corner anchored at the intersection of the lens and

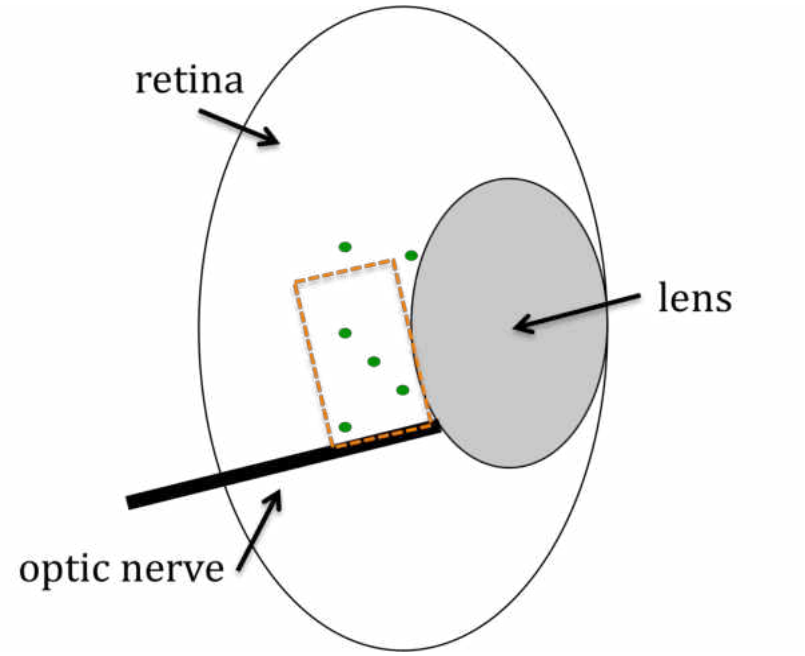

Figure 2. Diagram of retinal section cell counts of antibody labeled cells. Sections of retina were stained with antibodies for active caspase 3, BrdU, or Zn-5. Retinal sections were overlaid with a rectangle in Metamorph® and positive cells within the 300 μm^2 area were manually counted (orange rectangle). The rectangle was anchored with one side on the lens and perpendicular side on the optic nerve. Arrows point to retina, lens, and optic nerve.

optic nerve (Fig. 2). All cells within the boundary that were labeled with an antibody were selected manually and counted using the Methamorph® software. Those cells that were positive for antibody staining also have corresponding nuclear DAPI staining. The counts were then exported into Microsoft Excel for further analysis. Cell counts were combined from 5 replicates involving 5 to 7 individual embryos heatshocked at 28 hpf or 30 hpf. To determine if the cell counts were significantly differently from each other One-way ANOVA was performed using independent sample sets.

Western blot

Embryos were collected just prior to heatshock, as well as 1,2,4, and 6 hours after heatshock. Whole protein extracts were collected from embryos by homogenizing deyolked embryos in lysis buffer (25 mM Tris pH 8.0, 2 mM EDTA, 10% glycerol, 1% Triton-X 100). The extracts were then diluted into 2x Laemmli buffer with 5% 2-Mercaptoethanol. Extracts were denatured by boiling, and separated by size on a pre-cast BIO-RAD 12% SDS-PAGE gel (BIO-RAD, Hercules, CA). The SDS-PAGE gel was then blotted in a wet blot mini-gel BIO-RAD system onto PVDF membrane for 1 hour at 100V at 4°C. The membrane was blocked with 2.5% dry milk in wash buffer and then probed with anti-myc epitope tag antibody (1:1000)(Invitrogen) and anti-tubulin antibody (1:1000)(Sigma-Aldrich) diluted into 0.25% gelatin (Type A, Sigma-Aldrich) in TBS-T and incubated overnight at 4°C. A secondary HRP-conjugated goat anti-mouse antibody (1:2500) (Jackson ImmunoResearch, West Grove, PA) was diluted into 2.5% dry milk in TBST and incubated for 2 hours at room temperature. Membranes were washed 3 times with wash buffer after each antibody incubation for 10 minutes. The blot was developed using the chemiluminescence Clarrity™ Western ECL substrate (BIORAD) and imaged using a cooled CCD high-speed camera. We used Image Lab™ software (BIORAD) to measure the intensity of the staining on the western blot during acquisition of the Image. The measurement of intensity was conducted on both tubulin and myc antibody probed blots for the HSP-GAP43s42a-IRES-nucGFP and HSP-GAP43WT-IRES-GFP lines.

CHAPTER III

Disrupting GAP-43 phosphorylation in transient transgenic embryos disrupts RGC axon development

The GAP-43 protein is among the most abundant membrane-associated proteins in neuronal growth cones and is a prominent phosphorylation target of the signal transduction molecule, protein kinase C (PKC). Its expression pattern and known interacting protein partners places GAP-43 in the proper position to have a role in axon growth and guidance. A role for GAP-43 in the organization of the cytoskeleton of growing axons has been established both *in vitro* and *in vivo* (Shen et al. 2002, Meiri and Gordon-Weeks 1990). More specifically it has been demonstrated that GAP-43 phosphorylation by PKC enables GAP-43 to act as a lateral stabilizer of f-actin, while GAP-43 that is unphosphorylated at the PKC site has decreased affinity for f-actin and can even function to block polymerization of actin monomers (He et al. 1997, Nguyen et al. 2009). In cultured cells the PKC phosphorylation site appears to be essential for GAP-43 to direct the organization of the neuronal cytoskeleton in response to external signals (Korshunova et al. 2007, Widmer and Caroni 1993). The goal of our experiments is to further this research by

addressing if GAP-43 phosphorylation is essential for the axonal growth and guidance during development *in vivo*.

Axon behavior is the result of an orchestrated multistep process that requires the regulation of actin by actin-accessory proteins like GAP-43 in response to the external guidance and growth cues that activate signal transduction molecules. In the developing visual system RGCs emerge from the asymmetrical division of retinal precursors and RGC axons encounter guidance cues that influence their growth and trajectory. Axons that emerge from the zebrafish RGCs traverse the OFL, exit the retina, cross the optic chiasm, and enter into the contralateral optic tract. At each stage of RGC axon development, growth cones modulate their structure in response to external signals via proteins that regulate the cytoskeleton. Previous research indicates that GAP-43 regulates the actin cytoskeleton during differentiation of neurons and is required for axons to navigate decision points in the visual system (Gupta et al. 2010, Strittmatter et al. 1995). Our experiments focused on the developing visual system to assess the role of GAP-43 phosphorylation on the development of RGC and the extension of RGC axons when exiting the retina, crossing the optic chiasm, and entering into the optic tract.

We hypothesized that GAP-43 phosphorylation by PKC is required for proper guidance of RGC axons in the developing visual system. To disrupt phosphorylation of GAP-43 using a dominant negative method we overexpressed a mutant form of GAP-43 that cannot be phosphorylated in response to guidance cues encountered by RGC growth cones within the visual system. Previous experiments in GAP-43 knockout mice demonstrated that in the absence of GAP-43, axons of RGCs were

unable to properly respond to guidance cues and took aberrant trajectories while crossing the optic chiasm (Strittmatter et al 1995, Zhu and Julien 1999). Based on the GAP-43 knockout mouse phenotype and the established actin interactions of GAP-43 phosphorylation mutants *in vitro* (Maier et al. 1999,He et al. 1997), we predicted that disrupting GAP-43 phosphorylation would uncouple changes in the actin cytoskeleton from the external signals that activate PKC in RGC growth cones, ultimately resulting in axon guidance defects.

Results

Expression of GAP-43 proteins in transient transgenic zebrafish embryos

To determine how the phosphorylation of GAP-43 is involved in the growth and guidance of axons in the zebrafish visual system, we created plasmid constructs to express a phosphorylation-deficient mutant form of GAP-43. The specific mutation results in a single amino acid substitution of serine to alanine at position 42 (GAP43s42a). Serine 42 in the fish GAP-43 is equivalent to serine 41 in the mammalian homologues of GAP-43 (Leu et al. 2010). This mutation prevents the phosphorylation of GAP-43 by PKC and has been shown in cultured cells to decrease the ability of GAP-43 to bind and stabilize f-actin filaments (Meiri et al. 1996, He et al. 1997). We designed plasmid constructs that could express the phosphorylation-deficient GAP-43 under the regulation of two different promoters (Fig. 3). A *Takifugu rubripes* GAP-43 promoter was utilized to promote neuron specific expression in a developmental pattern similar to the expression of endogenous GAP-

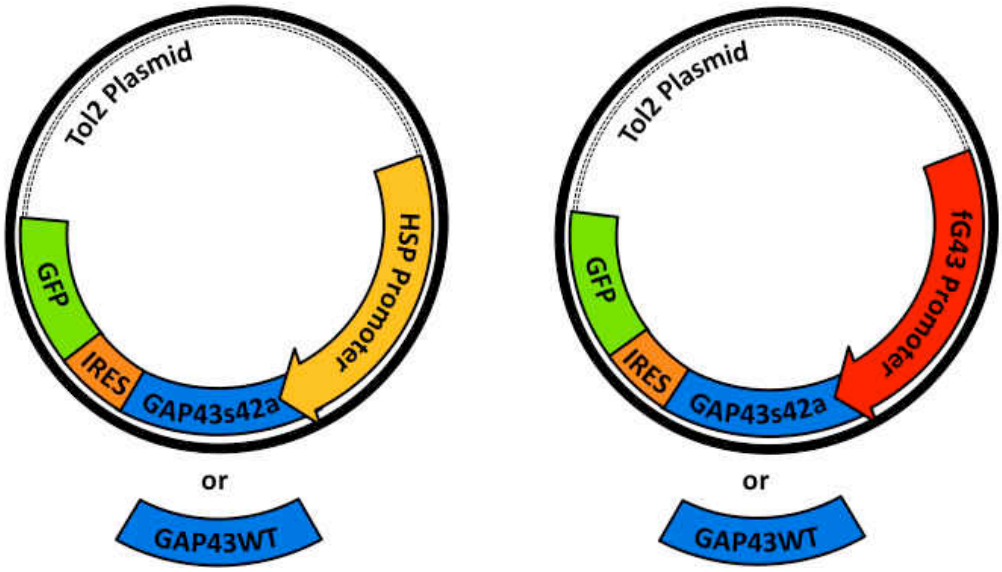

Figure 3. Plasmid constructs for the overexpression of mutant GAP-43. Plasmids were constructed to express a bicistronic mRNA encoding the mutant GAP-43 and GFP under the regulation of the HSP or *fugu* GAP-43 promoter. The internal ribosomal entry site (IRES) allows the GAP-43 and GFP cistrons to be independently translated. The HSP promoter induces expression of transgenes only after exposure to elevated temperatures (38°C for 1 hour). The *fugu* GAP-43 promoter drives expression of transgenes in developing and regenerating neurons in a spatial and temporal pattern that mimics endogenous GAP-43 expression.

43 (Udvadia 2008), and a heatshock protein (HSP) promoter was employed to establish heat-induced expression at different developmental times (Halloran et al. 2000). These plasmids are referred to as fG43-GAP43s42a-IRES-nucGFP and HSP-GAP43s42a-IRES-nucGFP, respectively.

Plasmids injected into the one cell-stage zebrafish embryos are known to segregate randomly among dividing cells resulting in embryos with mosaic expression of the transgene (Stuart et al. 1990). In order to identify plasmid containing cells in the injected embryo, we engineered the plasmid constructs to express a bicistronic mRNA that encodes the mutant GAP-43 protein as well as the nuclear or cytoplasmic directed jellyfish green fluorescent protein (GFP). GFP is translated separately from the GAP-43 using IRES element, from

encephalomyocarditis virus, that was cloned between the two coding sequences (Fig. 3). Plasmids with a similar design were created to express the wild type GAP-43 (fG43-GAP43WT-IRES-GFP or HSP-GAP43WT-IRES-GFP) in place of the mutant GAP-43 to serve as a control (Fig. 3).

While our ultimate goal was to develop transgenic lines of zebrafish that had stably integrated the plasmids into their germ cells, we wanted to determine if expression in the transient transgenic fish (i.e. fish injected at the 1-cell stage) was widespread and consistent enough to permit initial analysis of the constructs in the F0 generation. As mentioned above, plasmid injections into 1-cell stage embryos typically resulted in a random distribution of the plasmid into the cells of the developing embryos. We were able to detect cells that expressed the bicistronic transgenic mRNA by virtue of the fluorescence from the GFP protein that was coexpressed with GAP-43. At 24 hpf embryos injected at the 1-cell stage with reporter genes under the regulation of the *fugu* GAP-43 promoter (fG43) displayed GFP in a random assortment of neurons throughout the developing nervous system as was previously reported for this promoter (data not shown, Udvadia, 2008). In contrast, fish injected with reporter genes under the regulation of the inducible HSP promoter showed very little GFP expression prior to heatshock. Induction is generated in the HSP-GAP43s42a-IRES-nucGFP and HSP-GAP43WT-IRES-GFP injected embryos by heating the embryos to 38°C for 1 hour. After heatshock, widespread GFP expression was observed starting at 1 hour after induction, peaking at 4 hours, and detectable until 18 hours after induction (data not shown). Figure 4 illustrates the expression of GFP in a 48 hpf embryo that was injected at the 1-cell

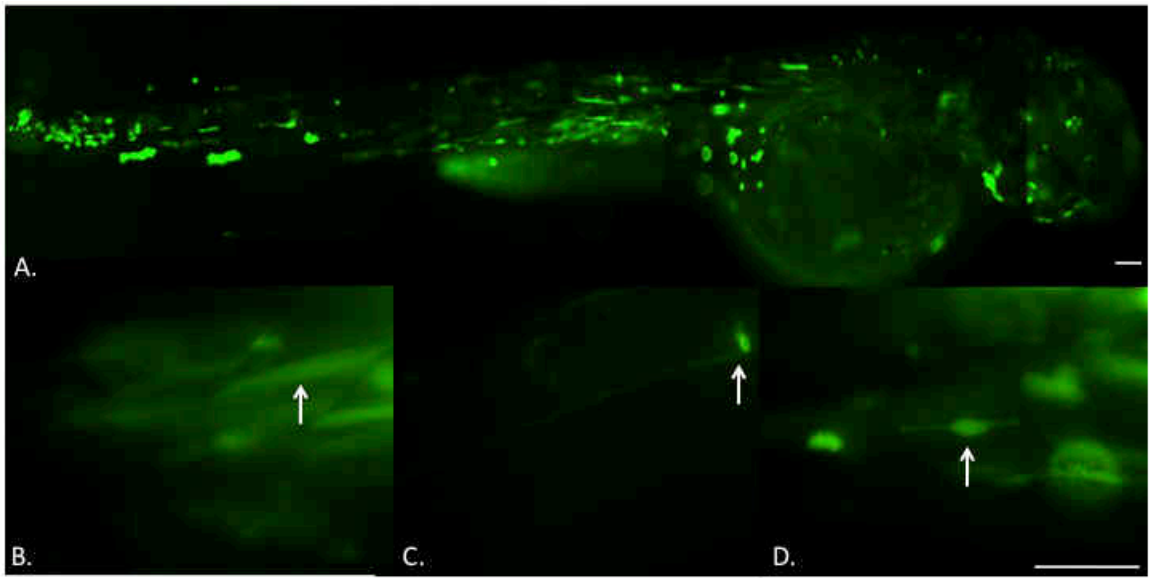

Figure 4. Widespread, mosaic expression of GFP in embryos injected with expression plasmids at the 1-cell stage. Image of mosaic transgene expression in 48 hpf embryos injected with HSP-GAP43WT-IRES-GFP and heatshocked at 30 hpf. (A). Image A is of the whole embryo. Expression of GFP was found in a variety of cells including: muscle cells (B) and neurons (C and D) (white arrows). The injected plasmid produced a bicistronic mRNA, encoding GAP43WT and GFP, allowing fluorescence from the GFP expression to be used as a marker of GAP43WT overexpression. The number of GFP expressing cells and the intensity of expression was heterogeneous. This widespread expression continued from 3 to 18 hours after heatshock and diminished over time. Images were acquired, compiled, and processed using Metamorph® software. Scale bars = 50µm. Scale bar in image D is representative for images B-D.

stage with the HSP-GAP43WT-IRES-GFP plasmid and was heatshocked at 30 hpf (18 hours of transgene induction). Expression was robust and distributed randomly among different cell types including muscle cells and neurons (Fig. 4B, 4C, and 4D). The high levels of GFP expression and the widespread distribution of expressing cells in the injected animals suggested that comparisons of mutant and wild type GAP-43 proteins could be carried out using transient transgenic assays.

Reduced survival of embryos expressing GAP43s42a during early nervous system development in transient transgenic embryo assays

To examine the role of GAP-43 phosphorylation in developing neurons we first examined embryos injected with the plasmid expressing the GAP43s42a phosphorylation mutant under the regulation of the *fugu* GAP-43 promoter in addition to endogenous GAP-43 expression. When the fG43-GAP43s42a-IRES-nucGFP was injected into embryos, we observed a significant reduction in the survival of injected embryos (Fig. 5). Only ~30% of embryos overexpressing GAP43s42a under the fG43 promoter survived from 24 hpf to 48 hpf. Although the reasons for the mortality of the fG43-GAP43s42a-IRES-nucGFP injected embryos have not been established, viability was unaffected in fG43-GAP43WT-IRES-GFP injected embryos (Fig. 5). Thus, it is likely that early expression of the mutant protein in the nervous system disrupted vital functions in the developing embryo. Given that the reduced viability of the embryos injected with fG43-GAP43s42a-IRES-nucGFP occurred during a stage when neurons in the developing visual system are first differentiating and extending axons, this expression system was not optimal to test our hypothesis regarding the role of GAP- 43 phosphorylation in

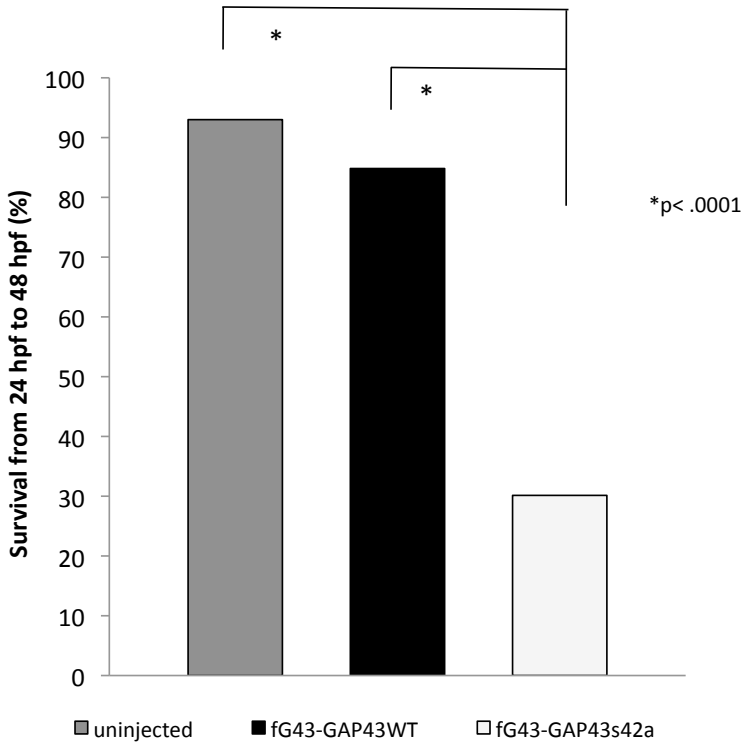

Figure 5. Expression of GAP43s42a in developing neurons results in reduced viability. Embryos were injected at the 1-cell stage with plasmids expressing GAP43s42a or GAP43WT under the regulation of the *fugu* GAP-43 promoter. Expression of GAP43s42a resulted in a significant reduction in the survival of embryos. In contrast, survival rates of embryos expressing GAP43WT did not differ significantly from uninjected control (heatshocked) embryos. Embryos were pooled from 2 injection replicates (Control n=200, GAP43WT n=112, GAP43s42a n=73). Significance was determined using a chi-square of association.

RGCs during axon growth and guidance.

Retinal ganglion cell axon growth and guidance defects in embryos with induced overexpression of GAP43s42a at 30 hpf

Since early expression of the phosphorylation deficient mutant GAP-43 protein was apparently deleterious to the embryos, we used the expression

plasmids regulated by the HSP gene promoter to gain temporal control over transgene expression. The use of the heat inducible promoter allowed us to delay transgene expression to a stage of development when the first retinal ganglion cells were beginning to extend axons, but after most early axonal tracts in the developing brain and spinal cord were established. Embryos in which GAP43s42a overexpression was induced at 30 hpf remained viable through adulthood and could be examined for defects in optic nerve development at later stages.

In order to visualize the effects of GAP43s42a overexpression on RGC axon development, these experiments utilized a previously established reporter line that expresses membrane targeted GFP in developing and regenerating neurons under the *fugu* GAP-43 promoter (Udvadia, 2008). In this reporter line at 48 hpf, a ventral view of the head shows a clear pattern of GFP in the cell membranes of retinal ganglion cells, with axons exiting the eye, crossing at the optic chiasm, and extending toward the midbrain on the contralateral side. Transgenes expressing GAP43s42a or GAP43WT under the HSP promoter were injected into the 1-cell stage embryos from the GFP reporter line, heatshocked at 30 hpf, and initially screened at 34 hpf for expression of the injected transgenes. The differentiated retinal ganglion cells of the reporter lines expressing membrane targeted GFP were easily distinguished from cells expressing the cytoplasmic GFP or nuclear GFP that was coexpressed with GAP43s42a or GAP43WT as part of the heatshock induced transgene cassettes. This distinction allowed us to select injected embryos at 34 hpf that were overexpressing the mutant or wild type GAP-43 specifically within the retina for later examination. Control embryos had membrane directed GFP

expression but were not injected with a HSP promoted plasmid and therefore did not express any additional transgenes. Selected embryos were imaged again at 48hpf and 72 hpf, when expression of the membrane targeted GFP (not part of the HSP transgenes cassette) reporter gene is robustly expressed in retinal ganglion cell axon, allowing us to easily visualize any deviations from the stereotypical path normally taken by the retinal axons. The GFP signal in the resulting images was derived from the membrane directed GFP under the fugu GAP-43 promoter. In contrast to uninjected control embryos or embryos overexpressing GAP43WT, we observed that embryos overexpressing GAP43s42a displayed defects in growth, fasciculation, and guidance of RGC axons when observed 18 and 42 hours after heatshock induction.

Initially at 48 hpf (18 hours after heatshock induction) the most prevalent defect was that the optic nerve appeared thinner. Compared to uninjected and GAP43WT overexpressing embryos, embryos in which GAP43s42a overexpression was induced at 30 hpf had a reduced diameter of the axon bundles within the optic nerve when visualized at 48 hpf (Fig. 6). This reduction was constant from the optic nerve stalk to the optic tract (Fig. 6B). The reduction in the diameter of the axon bundles could not be attributed to non-specific effects of injection or heatshock because similarly treated embryos overexpressing GAP43WT were indistinguishable from uninjected control embryos (Fig. 6A and 6C). Similarly, embryos that were injected with the HSP-GAP43s42a-IRES-nucGFP, but not induced to express GAP43s42a (not heatshocked), were indistinguishable from control. A qualitative assessment of these animals suggests that the reduction in optic nerve

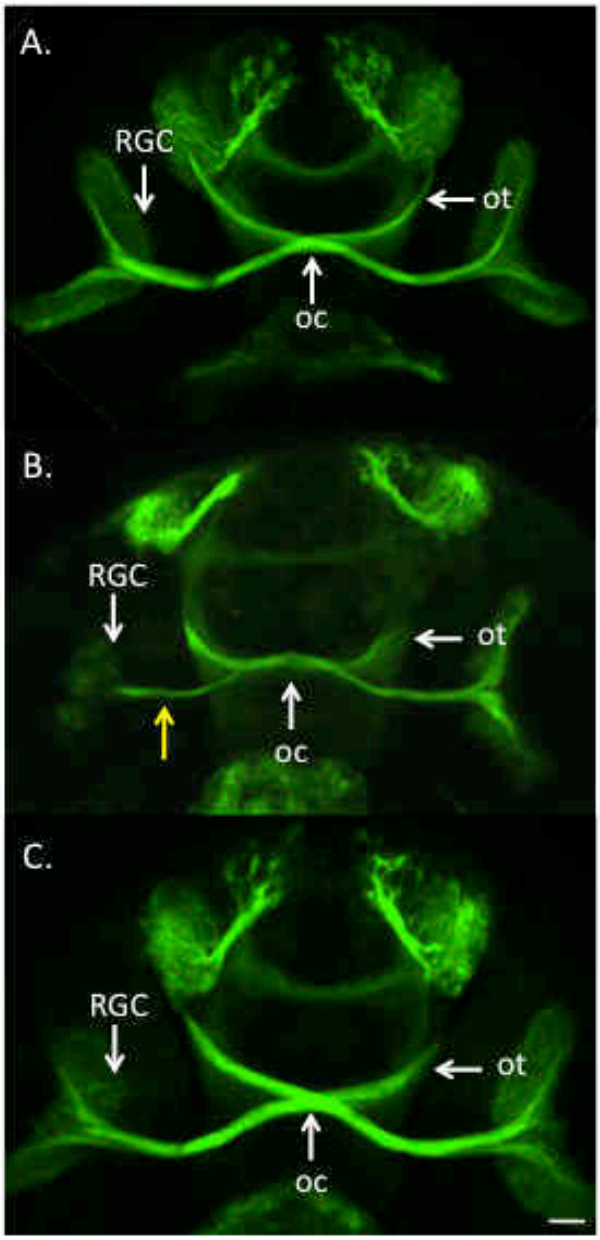

Figure 6. Decreased optic nerve diameter at 48 hpf in embryos induced to overexpress GAP43s42a at 30 hpf. Images are of 48 hpf embryos that were injected with HSP-GAP43s42a-IRES-nucGFP or HSP-GAP43WT-IRES-GFP and heatshocked at 30 hpf. Image A is a heatshocked unjected control embryo. Embryos in images B and C are injected and overexpressing GAP43s42a and GAP43WT respectively. In control embryos, RGC axons form a dense bundle to generate an optic nerve (A). In contrast the optic nerve in GAP43s42a overexpressing embryos has a reduced diameter (yellow arrow B). The optic nerve in embryos overexpressing GAP43WT is indistinguishable from the control optic nerve (C). Live embryos were mounted ventrally to visualize GFP expressing RGC axons extending from the RGC cell bodies (RGC) in the retina and crossing at the optic chiasm (oc) into the optic tract (ot). Confocal images were acquired, compiled, and processed using Metamorph® software. Scale bar = 50µm.

diameter is caused by a reduction in the number of axons comprising the optic nerve, which in turn could be due to either a failure or delay in RGC axon formation.

By 42 hours after heat induction (72 hpf) we observed a recovery in optic nerve diameter indicating that axon extension was not permanently inhibited. Instead at 72 hpf, the most prominent defect appeared in the compaction of axon bundles within the optic nerve. In comparison to control and GAP43WT overexpressing embryos, GAP43s42a overexpression resulted in an increased optic nerve diameter presumably due to an impaired ability of axons to associate with other axons (Fig. 7). In the zebrafish visual system RGC axons exit the retina in a staggered fashion after differentiation and the optic nerve is formed when newer axons associate with previously extended axons also called pioneer axons. Induced overexpression of GAP43s42a caused a disruption in this association, particularly in the posterior portion of the optic nerve tract (Fig. 7B). In comparison to the uneven association of axons in embryos overexpressing GAP43s42a, axons in the optic nerve of embryos overexpressing GAP43WT fasciculated normally and appear identical to uninjected but heat-treated control embryos (Fig. 7A and 7C). Defasciculation of the optic nerve can be caused by the inability of axons in embryos overexpressing GAP43s42a to rearrange the actin cytoskeleton in response to cell-to-cell adhesion cues or other guidance molecules that direct the bundling of axons within the optic nerve.

While variability in optic nerve diameter was the most abundant defect, instances of misguidance of RGC axons within the visual system occurred at both 48

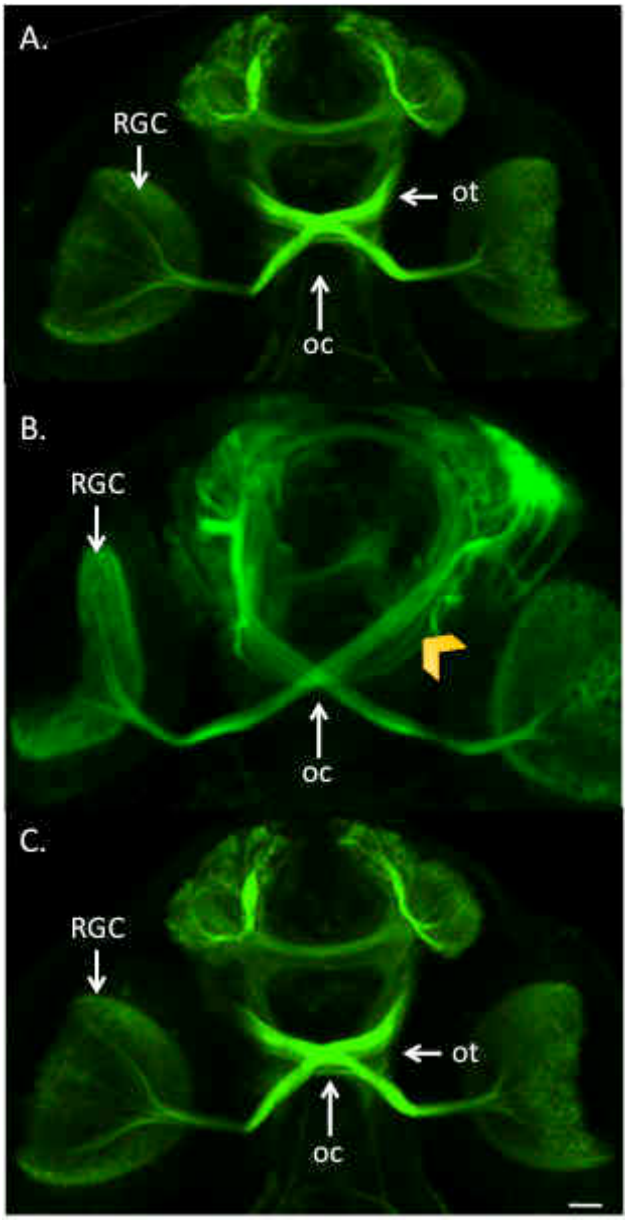

Figure 7. Axon defasciculation within the optic nerve at 72 hpf in embryos induced to overexpress GAP43s42a at 30 hpf. Embryos were injected with either HSP-GAP43s42a-IRES-nucGFP or HSP-GAP43WT-IRES-GFP and heatshocked at 30 hpf. A 72 hpf control embryo is displayed in image A. Embryos overexpressing GAP43s42a or GAP43WT are displayed in images B and C respectively. Uninjected control embryos displayed normal fasciculation of axons (A). Embryos overexpressing GAP43s42a displayed a defasciculated optic nerve at 72 hpf with ectopically spaced axons (yellow chevron B). Embryos overexpressing GAP43WT displayed a normally fasciculated optic nerve (C). Live embryos were mounted ventrally to visualize GFP expressing RGC axons extending from the RGC cell bodies (RGC) in the retina and crossing at the optic chiasm (oc) into the optic tract (ot). Confocal images were acquired, compiled, and processed using Metamorph® software. Scale bar = 50µm.

hpf and 72 hpf. In a small number of cases overexpression of GAP43s42a resulted in axons along the optic tract taking aberrant trajectories. In contrast, axon trajectory at both ages was normal in embryos overexpressing GAP43WT, which indicates that misguidance is not caused by non-specific effects of injection and heating. At 48 hpf, RGC axons in embryos overexpressing GAP43s42a deviated from the normal tract of the optic nerve (Fig. 8A). Axons in embryos overexpressing GAP43WT and control uninjected embryos grew normally and associated with other axons of the optic nerves, but in embryos overexpressing GAP43s42a axons appeared to disassociate and grow away from the optic nerve. When embryos overexpressing GAP43s42a were imaged at 72 hpf, RGC axons also displayed aberrant trajectories with the majority of the errors occurring after the transect at the optic chiasm (Fig. 8B and 8C). In injected embryos, the number of cells overexpressing GAP43s42a and the amount of GAP43s42a overexpression varied in each cell. This variation caused a range in the severity of guidance errors. In some embryos overexpressing GAP43s42a, individual axons strayed from the optic tract and headed back towards the midline (Fig. 8B.1) and in other rarer cases the whole optic nerve grew in ectopic directions (Fig. 8C.1). After crossing the optic chiasm GAP-43 phosphorylation is required for guidance of RGC axons along the optic tract.

Quantitatively embryos overexpressing GAP43s42a had significantly more defects in RGC axon growth and guidance than control embryos or those overexpressing GAP43WT. The defects included reduced optic nerve diameter, axon defasciculation, and abnormal axonal trajectory. After induction, most GAP43s42a

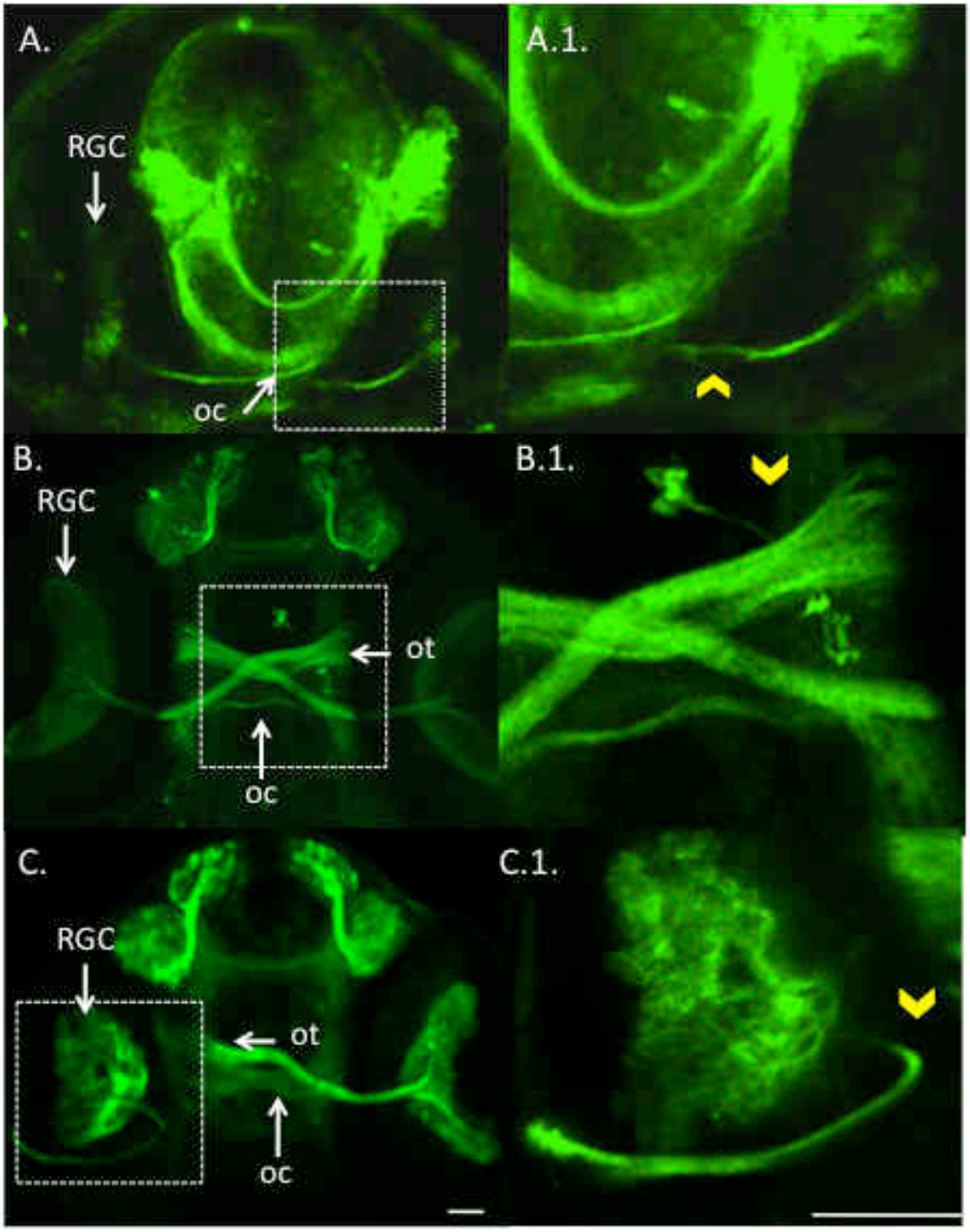
A.
RGC
oc
A.1.
B.
RGC
ot
oc
B.1.
C.
RGC
ot
oc
C.1.

Figure 8. Axon guidance errors at 48 and 72 hpf in embryos induced to overexpress GAP43s42a at 30 hpf. Embryos were injected with HSP-GAP43s42a-IRES-nucGFP than imaged at 48 hpf (A) or 72 hpf (B and C). At 48hpf, RGC axons overexpressing GAP43s42a show aberrant trajectories prior to reaching the optic chiasm (A) and under higher magnification axons deviate from the normal optic nerve course after exiting the retina (yellow chevron A.1). At 72 hpf, RGC axons overexpressing GAP43s42a deviate from the optic nerve in the optic tract (B) with individual axons taking aberrant trajectories (yellow chevron B.1). Also at 72hpf, RGC axons overexpressing GAP43s42a displayed severe guidance errors (C) where all axons within the optic nerve completely grow away from the optic chiasm (yellow chevron C.1). Variability in the numbers of axons taking abnormal trajectory was observed, which was not unexpected due to the mosaic distribution of transgene overexpression in injected embryos. Live embryos were mounted ventrally to visualize GFP expressing RGC axons extending from the RGC cell bodies (RGC) in the retina and crossing at the optic chiasm (oc) into the optic tract (ot). Confocal images were acquired, compiled, and processed using Metamorph® software. Scale bar = 50μm

overexpressing embryos showed defects in optic nerve formation, with 85% showing defects at 48 hpf, and 78% showing defects at 72 hpf (Fig 9 and 10). In contrast embryos overexpressing GAP43WT had significantly fewer defects at both 48 hpf (18.1%) and 72 hpf (15%) and were indistinguishable from control embryos, which were uninjected but exposed to heat.

These defects occurred in a temporal pattern with thinner optic nerves observed more frequently in younger (48 hpf) embryos overexpressing GAP43s42a, while defasciculation was more prominent in 72 hpf embryos overexpressing GAP43s42a. At 48 hpf, a reduced optic nerve diameter was the most abundant defect observed in embryos with GAP43s42a overexpression (60%) (Fig. 9). In comparison only 13.6% of embryos overexpressing GAP43WT and 4.5% of control embryos displayed a reduced optic nerve diameter at 48 hpf. At 48 hpf overexpression of GAP43s42a caused a significant increase in the number embryos with a reduced optic nerve diameter. In 48 hpf control embryos and embryos

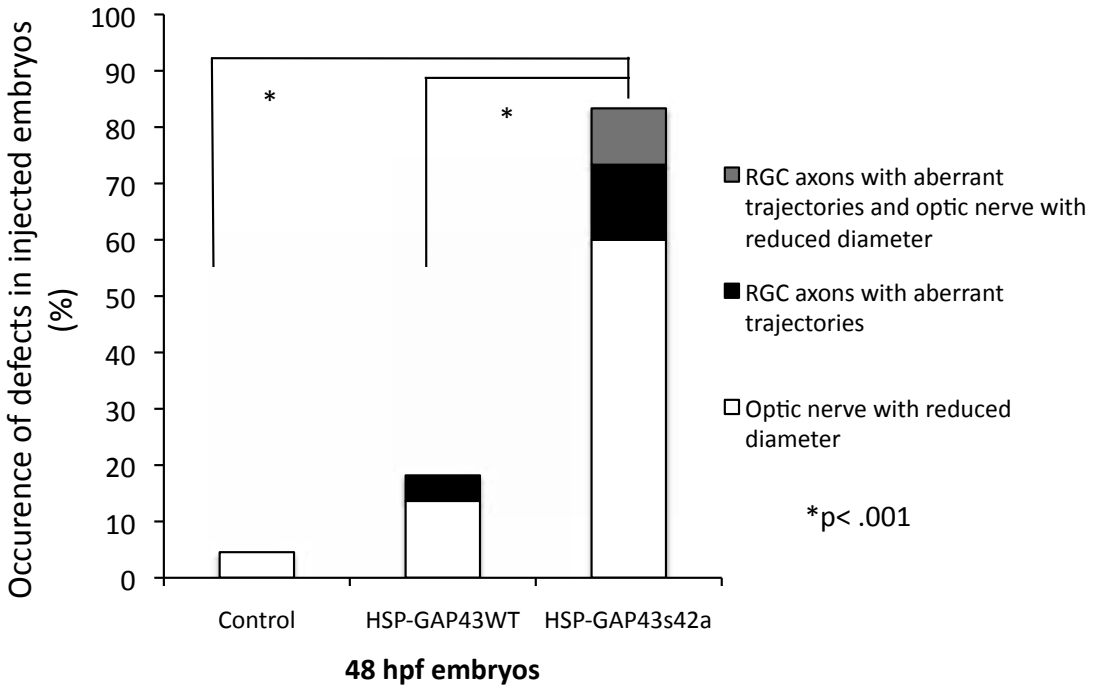

Figure 9. Reduced optic nerve diameter is the most prevalent axon defect at 48 hpf in embryos induced to overexpress GAP43s42a at 30 hpf. Almost 85% of embryos overexpressing GAP43s42a displayed RGC axonal defects, while embryos expressing GAP43WT did not differ from control embryos. The most prevalent defect observed at 48 hpf was a reduction in optic nerve diameter. Guidance errors occurred less often, and abnormal fasciculation was not observed. Embryos injected with HSP-GAP43s42a-IRES-nucGFP, HSP-GAP43WT-IRES-GFP or uninjected embryos were heatshocked at 30 hpf and mounted ventrally for confocal imaging of retinal axons at 48 hpf. Images were examined for a qualitatively abnormal optic nerve width, abnormal axonal fasciculation or trajectory. Graphs reflect the percentage of embryos from each group displaying 1) reduced optic nerve diameter, 2) guidance errors, and/or 3) abnormal fasciculation, and were pooled from embryos from 3 separate injections. (Control n=22, GAP43WT n=22, and GAP43s42a n=30). Significant differences in axonal defects were determined using the chi-squared test.

overexpressing GAP43WT there was no significant difference in the occurrence of defects ($p>0.1$). By 72 hpf only 9.1% of GAP43s42a overexpressing embryos displayed reduced optic nerve diameter, suggesting that axon outgrowth was temporally delayed, but not permanently inhibited by induction of GAP43s42a at 30hpf. Again at 72 hpf HSP-GAP43WT- IRES-GFP injected embryos did not differ significantly from control embryos with regard to the number of individuals with a reduced optic nerve ($p>0.1$)

Although axon outgrowth from RGCs appeared to recover by 72 hpf, the topography of axons in the optic nerve was still abnormal in embryos overexpressing GAP43s42a compared to control embryos or those overexpressing GAP43WT. Defasciculation of axons in the optic nerve was the most abundant defect observed at 72 hpf with 45% of GAP43s42a overexpressing embryos having a defasciculated optic nerve and 5% having both a defasciculated optic nerve and RGC axons guidance errors (Fig. 10). Defasciculation was not observed in the 48 hpf embryos. In comparison none of the embryos overexpressing GAP43WT displayed defasciculated axons in the optic nerve at 48 hpf and only 10% of both GAP43WT overexpressing embryos and control embryos had defasciculated axons in the optic nerve at 72 hpf.

Axons with aberrant trajectories in the optic chiasm occurred less often than defects in differentiation and defasciculation. However, more errors in axon trajectory were observed at 72 hpf than at 48 hpf. 18.18% of 72 hpf embryos expressing GAP43s42 displayed axons guidance defects, compared to 13.3% at 48 hpf (Fig. 9 and 10). At 48 hpf, 10% of embryos overexpressing GAP43s42a had both a reduced optic nerve diameter and RGC axons with abhorrent trajectories. In contrast, only 5% of embryos overexpressing GAP43WT displayed guidance errors at 48 hpf and at 72 hpf guidance errors were not observed. Guidance errors were not observed at either time point in control embryos. The difference in the occurrence of guidance errors between GAP43WT overexpressing embryos and control embryos was not significant at either 48 hpf or 72 hpf ($p>0.5$ and $p>0.1$).

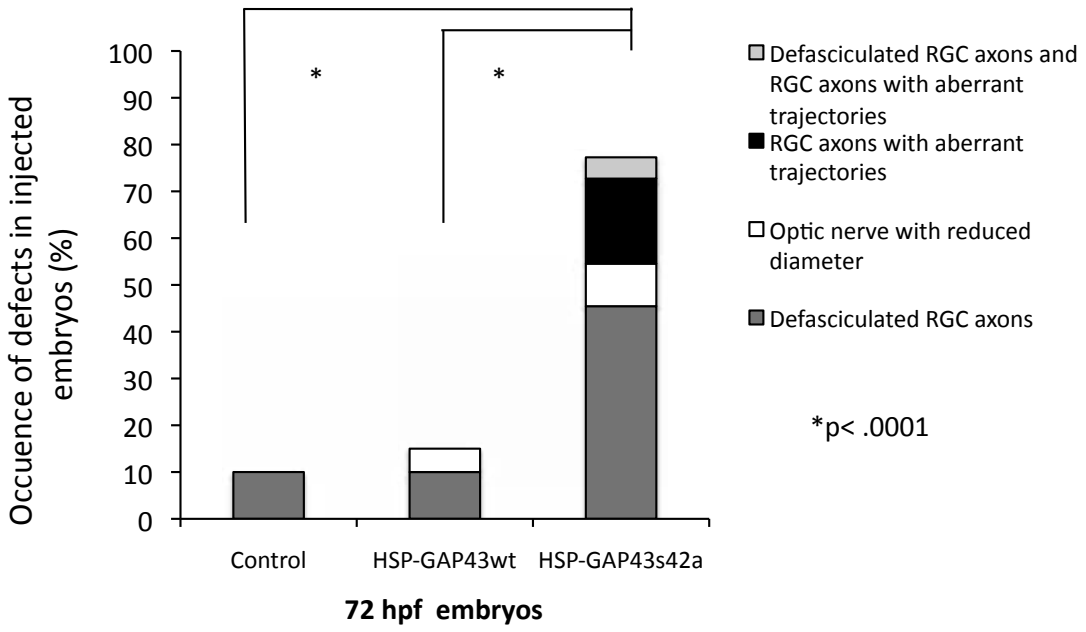

Figure 10. Axon defasciculation is the most abundant defect at 72 hpf in embryos induced to overexpress GAP43s42a at 30 hpf. 78% of embryos overexpressing GAP43s42a displayed RGC axonal defects, while embryos overexpressing GAP43WT did not differ from control embryos. The most prevalent defect observed at 72 hpf was a defasciculated axon bundles in the optic nerve. Guidance errors were the second most abundant defect observed, and a reduced optic nerve diameter was the least abundant. Embryos injected with HSP-GAP43s42a-IRES-nucGFP, HSP-GAP43WT-IRES-GFP or uninjected embryos were heatshocked at 30 hpf and mounted ventrally for confocal imaging of retinal axons at 72 hpf. Optic nerves were qualitatively examined for abnormal axonal fasciculation, abnormal optic nerve diameter or trajectory. Graphs reflect the percentage of embryos from each group displaying 1) reduced optic nerve diameter, 2) guidance errors, and/or 3) abnormal fasciculation, and were pooled from embryos from 3 separate injection experiments (Control n=22, GAP43WT n=22, GAP43s42a n=20). Significant differences in axonal defects were determined using the chi-squared test.

Conclusions and Summary

When GAP43s42a was overexpressed during the start of visual system development we observed defects in axon diameter including both a reduced optic nerve at 48 hpf and a defasciculated optic nerve at 72 hpf. Less frequently GAP43s42a overexpression caused misguidance of RGC axons during navigation of the developing visual system. Previous experiments demonstrated that the GAP43s42a phosphorylation mutant does not stabilize actin in a manner similar to endogenous phosphorylated GAP-43 (Widmer and Caroni 1993). The RGC defects we observed after the overexpression of the GAP43s42a mutant are consistent with the loss of GAP-43 actin stabilization during different aspects of RGC axon development including neuronal precursor differentiation, axon guidance, and axon fasciculation.

At 48 hpf, the overexpression of GAP43s42a caused a reduction in the optic nerve diameter, which could be the result of fewer axons comprising the optic nerve or fewer RGCs. Defects during RGC axon guidance in the retina could delay or prevent RGC axons from exiting the retina and reduce the number of axons comprising the optic nerve. Overexpression of an unphosphorylatable GAP-43 in growth cones could disrupt the signaling cascade that normally results in changes in the cytoskeleton by replacing endogenous GAP-43 with GAP43s42a at actin binding sites. Consequently when phosphorylated GAP-43 is downstream of guidance molecules that are essential for axons exiting the retina, like NCAM, removing the phosphorylation of GAP-43 could mimic the removal of the guidance cues and result in the failure of RGC axons to exit the retina (Zelina et al. 2005, Meiri et al. 1998).

In a similar manner, GAP43s42a competing with endogenous phosphorylated GAP-43 during the differentiation of retinal ganglion cell precursors could disrupt the known role of GAP-43 in organizing cellular components during asymmetrical cell division (Gupta et al. 2010). Halting differentiation and reducing the number of mature RGCs would also reduce the number of axons in the optic nerve. Further investigation is needed to determine exactly how each possibility contributes to a reduced optic nerve diameter.

At 72 hpf, an increased diameter of the optic nerve could be the result of a failure of RGC axons overexpressing GAP43s42a to fasciculate with other axons in the optic nerve. Similar to the proposed mechanism in the retina, overexpressing GAP43s42a when axons are associating in the optic chiasm could uncouple fasciculation cues from changes in the actin cytoskeleton. This inability to respond to fasciculation cues like NCAM could result in a defasciculated optic nerve (Rutishauser et al. 1988).

Similar to an inability to respond to fasciculation cues within the optic chiasm, embryos overexpressing GAP43s42a could also be unable to correctly regulate actin dynamics in response to guidance cues in the optic chiasm. Previous research indicates that GAP-43 is required for RGC axons to navigate the optic chiasm. Disrupting GAP-43 phosphorylation could also lead to axon guidance defects if phosphorylation regulates GAP-43 activity (Zhu and Julien 1999). Axon guidance defects at 48 hpf and 72 hpf in GAP43s42a overexpressing RGC axons is consistent with a failure to properly organize the actin cytoskeleton in response to guidance cues.

As described above a reduced optic nerve diameter was observed extensively in GAP43s42a embryos at 48 hpf. By 72 hpf the reduction of the optic nerve diameter was significantly less prevalent in GAP43s42a overexpressing embryos. By 72 hpf axons outgrowth could have recovered significantly enough to no longer cause a reduced diameter. While a single pulse of GAP43s42a expression was sufficient to cause a reduction in diameter at 48 hpf, it may have been insufficient to cause a significant reduction of the diameter of the optic nerve at 72 hpf. Degradation of GAP43s42a at later ages could also facilitate the recovery of RGC axons from the delayed axon outgrowth.

In contrast, defasciculation of the optic nerve occurred predominantly in 72hpf GAP43s42a overexpressing embryos. Defasciculation was not observed in 48hpf embryos overexpressing GAP43s42a. The absence of fasciculation defects at 48hpf was most likely due to the delayed axons outgrowth from the retina caused by GAP43s42a overexpression. A delay in outgrowth from the retina would delay the fasciculation of axons. When axons recovered and eventually extended, defects in their ability to fasciculate became apparent when imaged at 72 hpf. Another possibility is that axon outgrowth is permanently inhibited but defasciculation effectively masks a reduction in the number of RGC axons.

Without further experimentation we are unable to determine systematically if early defects in visual system development elicits later defects or if both are independently caused by GAP43s42a overexpression. Errors made during retinal guidance by RGC growth cones could change the local or temporal growth environment of RGC axons in the optic chiasm causing guidance and fasciculation

errors. Also possible is an independent role for phosphorylated GAP43 in the differentiation of neurons in the retina, and in the guidance and fasciculation of RGC axons in the optic chiasm. Experiments described in the next chapter focus on each of these effects individually to determine the exact role of GAP-43 phosphorylation.

CHAPTER IV

Disrupting GAP-43 phosphorylation in stable transgenic embryos disrupts RGC differentiation, guidance and fasciculation

In the transient transgenic experiments described in the previous chapter, the overexpression of GAP43s42a disrupted the formation of the optic nerve in a developmental stage specific manner, indicating a different role for phosphorylated GAP-43 at two distinct stages of RGC axon development. 48 hpf embryos overexpressing GAP43s42a had a reduced optic nerve diameter. In contrast 72 hpf embryos overexpressing GAP43s42a displayed defects in the fasciculation of axon bundles during optic nerve formation in the optic chiasm. Overexpression of GAP43s42a caused guidance errors to occur in both 48 hpf and 72 hpf embryos, although at a lower rate than the defects in optic nerve diameter. These results suggest that phosphorylated GAP-43 might have a unique role during the two distinct stages of RGC development. In order to validate and extend these results we have created stable transgenic lines that inducibly overexpress mutant or wild type GAP-43 in a uniform manner compared with the injected animals, which display mosaic expression. These animals will allow us to more precisely quantify the role of GAP-43 phosphorylation at different stages of RGC development.

During the earliest stages of visual system development retinal precursors differentiate into RGCs and extend axons that must reach the optic nerve head to exit the retina. Overexpression of GAP43s42a during this stage reduced the diameter of the optic nerve presumably by reducing the number of axons. We hypothesize that the overexpression of GAP43s42a during early visual system development, disrupts GAP-43 activity in asymmetrically dividing precursors described previously in cerebellar granule cells (Gupta 2010, Mishra et al 2008). Another explanation is that phosphorylated GAP-43 is required for RGC axons to respond to cues that guide axons to exit the retina at the optic nerve head. Both scenarios would reduce the number of axons within the optic nerve.

Defasciculation of the optic nerve and aberrant RGC trajectories in embryos overexpressing GAP43s42a are also consistent with a failure to respond to guidance cues. If RGC axons overexpressing GAP43s42a fail to regulate actin organization in response to cues like NCAM that direct fasciculation, the resulting defect would be defasciculation of the optic nerve. Similarly if RGC axons overexpressing GAP43s42a fail to regulate the actin cytoskeleton in response to guidance cues, this would result in RGC axons taking aberrant trajectories during navigation of the optic chiasm. Another possibility is the both fasciculation and guidance cures are required for the formation of the visual system. In each case it is possible that earlier defects in axons exiting the retina could disrupt the timing essential for fasciculation and guidance at the optic chiasm. To determine if the defects are linked or independent we used the inducible HSP promoter to temporally target the overexpression of GAP43s42a to specific stages in RGC axon development.

In these experiments expression of the phosphorylation mutant was induced either during early RGC development in the retina (28 hpf, 30 hpf), or later when RGC axons are approaching or crossing the optic chiasm (32 hpf, 34 hpf, 36 hpf)(see Fig. 1). We predicted that overexpression of GAP43s42 in stable transgenic embryos during differentiation and axon extension within the retina (28 hpf or 30 hpf) would result in a reduced optic nerve diameter. In contrast, we expected that overexpression of GAP43s42 later in development, during axon guidance and fasciculation in the optic chiasm (32 hpf, 34 hpf, or 36 hpf), would result in optic nerve defasciculation and guidance errors within the optic chiasm. Regulating the induction of the phosphorylation mutant with the inducible promoter and labeling axons with a fluorescent antibody for a neuronal membrane marker (Zn-5) allowed us to more precisely quantify the defects associated with the perturbation of GAP-43 phosphorylation during different stages of RGC axons development.

Results

HSP promoter drives robust expression of transgene in stable lines

In order to visualize the expression dynamics of the biscistronic message after heatshock induction, we created a control transgenic line, HSP-nucmCherry-IRES-GFPCAXX line, in which nuclear-targeted mCherry replaced the GAP-43 component and membrane-targeted GFP was expressed from the IRES of the previously described transgene constructs (Fig. 3). The expression of fluorescent reporter proteins in these embryos was expected to be representative of transgene expression in our wild type and mutant GAP-43 transgenic lines. We therefore

determined the efficiency of induction and expression in our HSP-driven transgenic lines using this fluorescent protein reporter. Figure 11 documents the abundant induction of both fluorescence proteins after heatshock (38°C) in stable lines of transgenic embryos. Heat induced the expression of fluorescent proteins in all cell types and including uniform expression throughout the nervous system due to the stable integration of the HSP-nucmCherry-IRES-GFPCAXX transgene into all cells (Fig. 11C, 11D, and 11E). We found little to no expression of the transgenes in the absence of heat induction, which ensured our ability to effectively control the timing of transgene expression. Fluorescence in the embryo without heatshock is the result of auto-fluorescence from the yolk. Figure 12 illustrates the time course of fluorescent protein induction after heatshock in embryos expressing HSP-nucmCherry-IRES-GFPCAXX. Over the course of 4 hours the expression of the mCherry protein in the head of the embryo rapidly increased (Fig. 12, similar timing of expression was observed for GFPCAAX), with the transgene barely detectable by 1 hour after induction, and the peek of expression at 4 hours after induction. By extension we expected that induction of the GAP-43 transgenes would occur along a similar timeline.

To document the induction of the GAP-43 transgenes, a western blot was performed on protein lysates from embryos from the stable transgenic lines. The western blot documents the induction of GAP43s42a and GAPWT from 0 to 6 hours after heatshock. To distinguish the transgenes from endogenous GAP-43, a myc epitope tag was incorporated at the 3'prime end of GAP43s42a and GAP43WT gene.

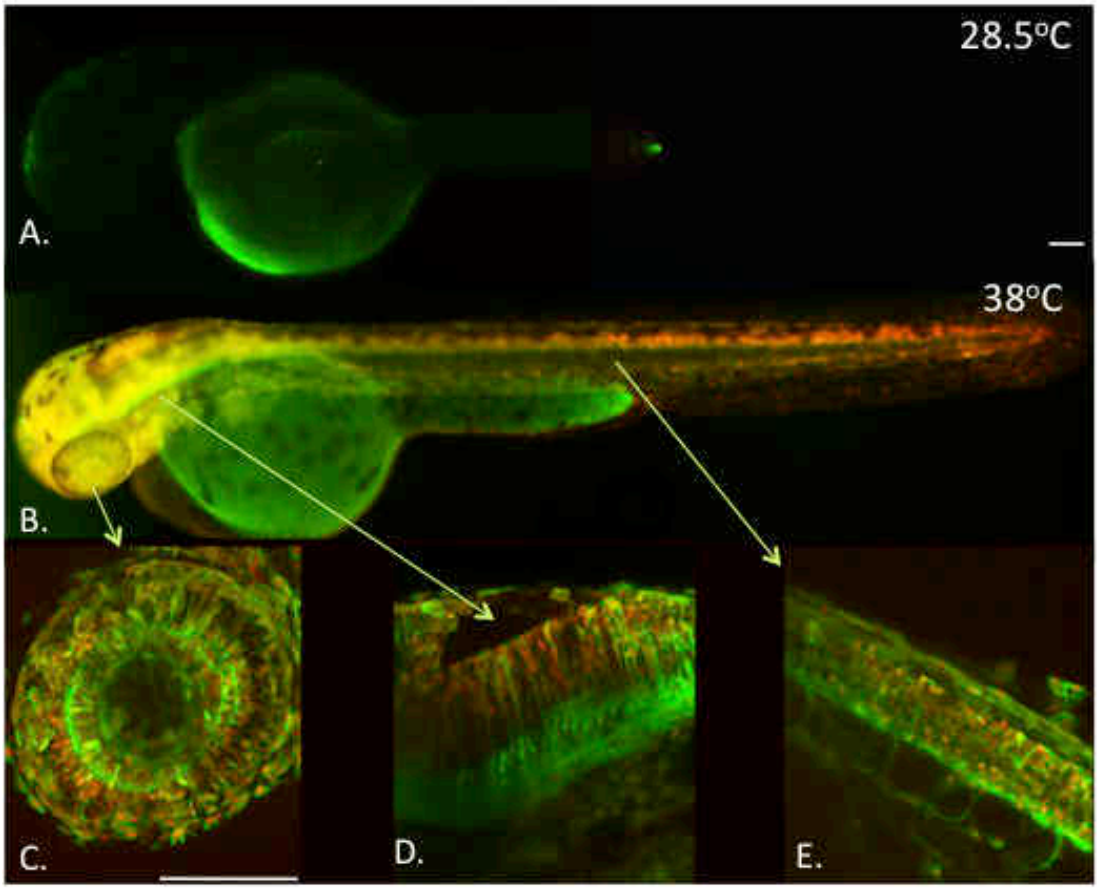

Figure 11. 48 hpf Embryo expressing HSP-nucmCherry-IRES-GFPCAXX post heatshock in all cells. Images are of embryos from the HSP-nucmCherry-IRES-GFPCAXX line both before heat shock (A) and 3 hours after heat shock at 38°C (B). The fluorescent nucleus directed mCherry and membrane direct GFP proteins were expressed through out the developing nervous system including the retina (C), hindbrain (D) and notochord (E). Live embryos were mounted laterally to visualize mCherry and GFP expression. In the stable lines heat induction causes transgene expression in all cells. In the embryo before heat shock the yolk displays auto-fluorescence. After heat shock cell bodies are labeled red with mCherry fluorescent protein and the membranes are labeled with green with GFP fluorescent protein. Confocal images were acquired, compiled, and processed with Metamorph® software. Scale bars =50µm. Scale bar in image A applies to images A and B. Scale bar in image C applies to images C-E.

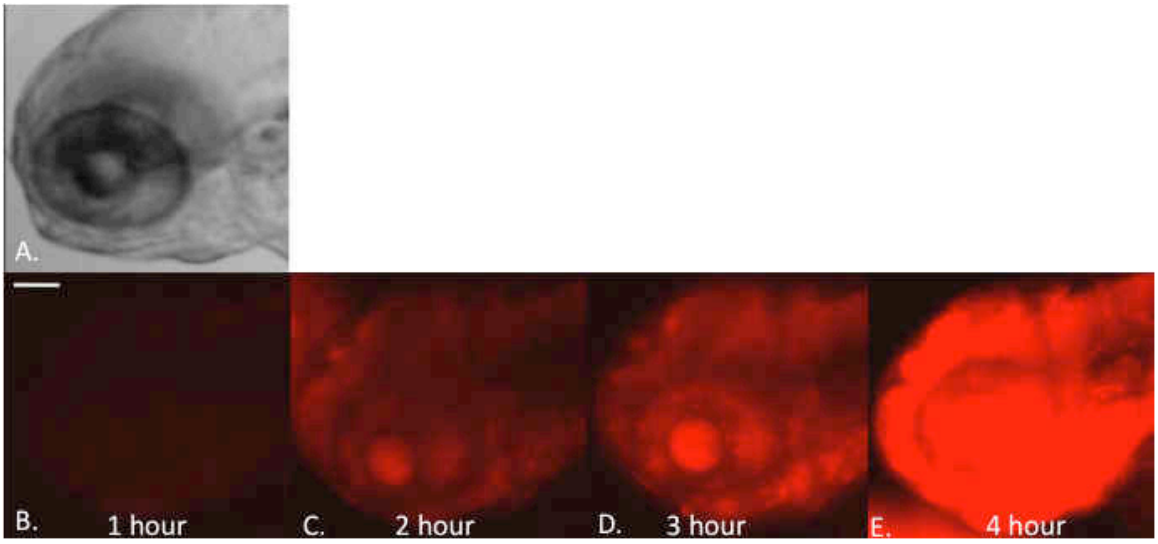

Figure 12. Fluorescent protein expression increases over time in 72 hpf embryos heatshocked to induce expression of nucmCherry. The images show the sequential progression of nuclear directed mCherry expression in the head of the same 72 hpf embryo (A, brightfield). Initially there are low levels of expression 1 hour after heat shock (B) and this progression of expression continues until it reaches high levels at 4 hr (E). Expression of the fluorescent protein reduces after 4 hours and is completely lost by 12 hpf (data not shown). Embryos were mounted laterally and immobilized in agarose to visualize fluorescent protein expression. This set of pictures demonstrates the robust expression of transgenes in the stable line after heatshock. Fluorescent images were acquired, compiled, and processed with Metamorph® software. Scale bar=50μm.

Protein lysates were collected from embryos and the induction of the transgene was detected with a myc antibody (Fig. 13). Starting at 1 hour after heat induction and continuing through 4 hours after induction, the expression of GAP43s42a and GAP43WT increased. The graph depicts the ratio of the optical density of the myc immunoreactive bands to the optical density of the control tubulin immunoreactive bands. The timing of induction of GAP43s42a and GAP43WT proteins matches the induction of the nucmCherry fluorescent protein (Fig. 12 and 13). Thus we confirmed that the HSP-driven transgene-encoded proteins have a peak accumulation at 4 hours after induction and subsequently show a rapid decrease.

Inducing overexpression of GAP43s42a early in RGC development caused a reduction in optic nerve diameter

Inducing GAP43s42a expression in the HSP-GAP43s42a-IRES-nucGFP transgenic line during the earlier stages of visual system development resulted in a significant reduction in the diameter of the optic nerve. The reduction is clearly noticeable when viewed but also apparent when the diameter of the optic nerve was measured. Disrupting GAP-43 phosphorylation during RGC differentiation, RGC axon extension, and navigation of the retina resulted in a significant reduction in the diameter of the optic nerve. When compared to embryos overexpressing GAP43WT, embryos overexpressing GAP43s42a showed a reduction in the optic nerve diameter when expression was induced at 28 hpf (Fig. 14A and 14B). To quantify the reduction associated with heatshock at 28 hpf, we immunostained embryos at 48 hpf with Zn-5 (pan-neuronal membrane marker), and measured the width of the optic nerve. Measurements were taken at two places: 1) directly after exiting the

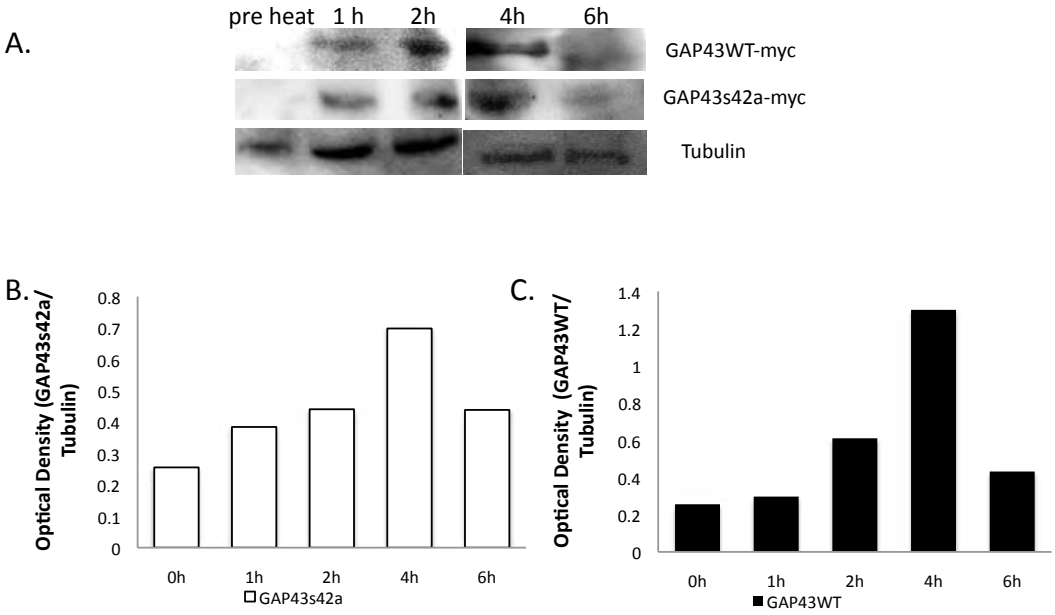

Figure 13. Western blot showing induction of myc tagged GAP43s42a and GAP43WT proteins. The western blot was conducted on protein lysates from GAP43WT and GAP43s42a overexpressing embryos. Blots were probed with myc and tubulin antibodies and the images are of GAP43s42a-myc, GAP43WT-myc and an example anti-tubulin blot (A). Only one tubulin blot is shown but there was one conducted for both GAP43s42a and GAP43WT protein lysates. Graphs of the optical density show that relative to the optical density of anti-tubulin probed bands the optical density of the anti-myc probed bands increased after induction till a peak at 4 hours (B). The same is true when comparing the bands of GAP43WT protein blots probed with anti-myc to those probed with anti-tubulin (C). Pre-heatshock embryos expressed very little GAP43WT or GAP32s42a and after heatshock expression increases until 4 hours. Blot pictures were taken over 30 seconds with a cooled CCD camera. The optical density measurements were taken with Image Lab software. The graphs are a ratio of the density of the anti-myc labeled bands compared to anti-tubulin labeled bands. Each lane contains approximately 50ng of protein.

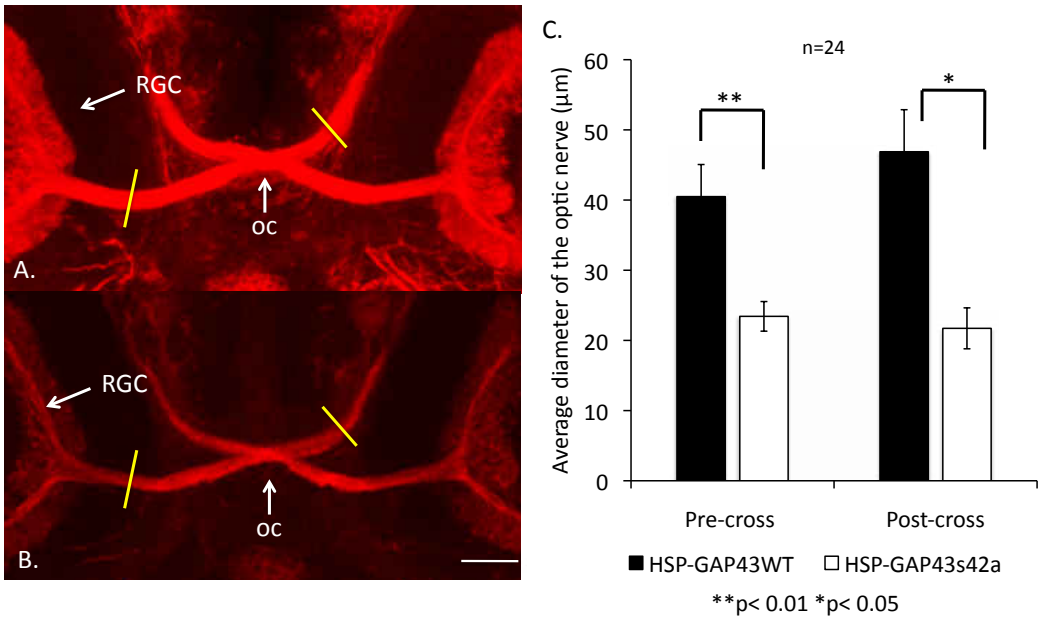

Figure 14. Reduced optic nerve diameter in the visual system of 48 hpf embryos induced to overexpress GAP43s42a at 28 hpf. Embryos overexpressing GAP42s42a starting at 28 hpf (B) had a reduced optic diameter in comparison to embryos overexpressing GAP43WT (A). The reduction was continuous throughout the entire length of the optic nerve. When the optic nerve was measured both before and after (yellow lines) transecting the optic chiasm (white arrow) there was a significant reduction in the diameter of the optic nerve (C). Fixed and immunostained (Zn-5 antibody) 48 hpf embryos were mounted ventrally in glycerol for visualization and measurements of axons as they extend from retinal ganglion cell bodies (RGC) into the optic chiasm (oc). Confocal images were acquired, compiled, and processed with Metamorph® software. Scale bars =50µm. Error bars are standard error, and these measurements were pooled from 4 replicates of the experiment (embryos n=24). Significance was calculated using One-way ANOVA.

retina, and 2) after transecting the optic chiasm. When GAP-43 phosphorylation was disrupted at 28 hpf with GAP43s42a overexpression, the diameter of the optic nerve prior to crossing the optic chiasm, compared to GAP43WT embryos (also induced at 28 hpf) was significantly reduced [GAP43s42a average diameter = 23.4±4.6 μm and GAP43WT average diameter =40.5±2.1μm] ($p<0.01$). The post-cross diameter of the optic nerve in GAP43s42a overexpressing embryos was also significantly smaller than the diameter of the GAP43WT overexpressing embryos [GAP43s42a average diameter = 21.7±2.9μm and GAP43WT average diameter = 46.9±6.0μm] ($p<0.05$).

Similar to results in the injected transient embryos, induction of GAP43s42a at 30 hpf also caused a reduction in the diameter of the optic nerve that was readily apparent when compared to the diameter of the optic nerve in GAP43WT overexpressing embryos (Fig. 15A and 15B). The labeled axons in embryos induced at 30 hpf were measured using the previously mentioned method. A reduction in pre-optic chiasm diameter was apparent in embryos induced to overexpress GAP43s42a at 30 hpf and this reduction was significant when compared to the diameter of embryos overexpressing GAP43WT [GAP43s42 average diameter = 26.9±4.7μm and GAP43WT average diameter = 40.3±2.8μm]($p<0.0001$). This also was true after crossing the optic chiasm, when GAP43s42a and GAP43WT was induced at 30 hpf, the post-cross diameter of the optic nerve was less in the GAP43s42a overexpressing embryos [GAP43s42a average diameter =23.6±2.8 μm and GAP43WT average diameter = 35.2±2.5μm] ($p<0.01$). From this quantification of the optic nerve diameter we concluded that expression of GAP43s42a during

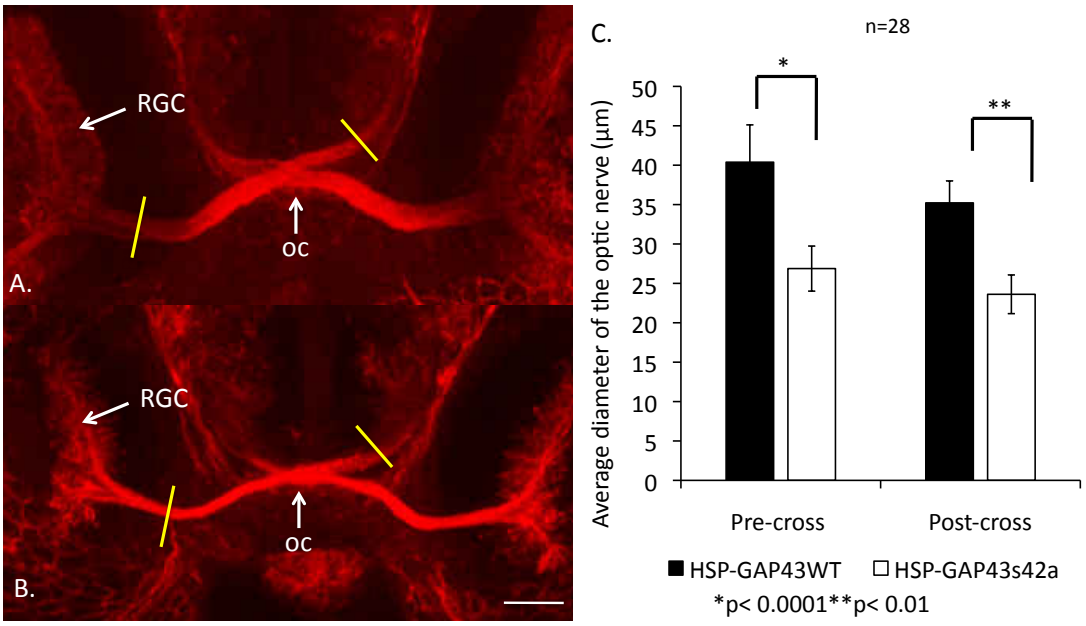

Figure 15. Reduced optic nerve diameter in the visual system of 48 hpf embryos induced to overexpress GAP43s42a at 30 hpf. Embryos overexpressing GAP42s42a starting at 30 hpf (B) had a reduced optic diameter in comparison to embryos overexpressing GAP43WT (A). The reduction was continuous throughout the entire length of the optic nerve. When the optic nerve was measured both before and after (yellow lines) the crossing at the optic chiasm (white arrow) there was a significant reduction in the diameter of the optic nerve (C). Fixed and neuronal membrane labeled (Zn-5 antibody) embryos were mounted ventrally in glycerol for visualization and measurements of axons as they extend from retinal ganglion cell bodies (RGC) into the optic chiasm (oc). Confocal images were acquired, compiled, and processed with Metamorph® software. Scale bars=50µm. Error bars are standard error and these measurements were pooled from 4 replicates of the experiment (embryos n= 28). Significance was calculated using One-way ANOVA.

early visual system development caused a reduction in the width of the optic nerve.

Induction of GAP43s42a reduced the population of retinal ganglion cells in the developing retina

A reduction in optic nerve diameter could indicate a possible reduction in the number of axons and mature RGCs. The retinas from embryos overexpressing GAP43s42a were immunostained with Zn-5, which labels differentiated RGCs in the retina, to determine if there was a reduction in the number of RGCs. After induction at 28 hpf or 30 hpf, embryos were allowed to develop until 48 hpf and then preserved, sectioned and immunostained. We compared the number of Zn-5 positive cells in embryos induced at 28 hpf or 30 hpf and analyzed at 48hpf. We compared cells from a selected portion of the retinal sections to analyze the same population of cells in each retina (see Fig 2). This survey of mature RGCs cells in GAP43s42a overexpressing embryos indicated that the reduction in optic nerve diameter was consistent with a reduction in the number of RGCs (Fig. 16). Induction at 28 hpf caused a reduction in the number of Zn-5 positive cells in GAP43s42a embryos [GAP43s42a had an average of 41.2±6.7 positive cells and GAP43WT had an average of 69.3± 3.6 positive cells] ($p<0.001$). In the 30 hpf-induced embryos there was also a reduction in the RGC population of GAP43s42a retinas compared to the population in the GAP43WT retinas [GAP43s42a had an average of 63.2± 2.2 positive cells and GAP43WT had an average of 74.4± 2.2 positive cells] ($P<0.001$). The reduction in the population of RGCs was less in embryos induced at 30 hpf (15% reduction) than the reduction observed in embryos when GAP43s42a was induced at 28 hpf (40% reduction).

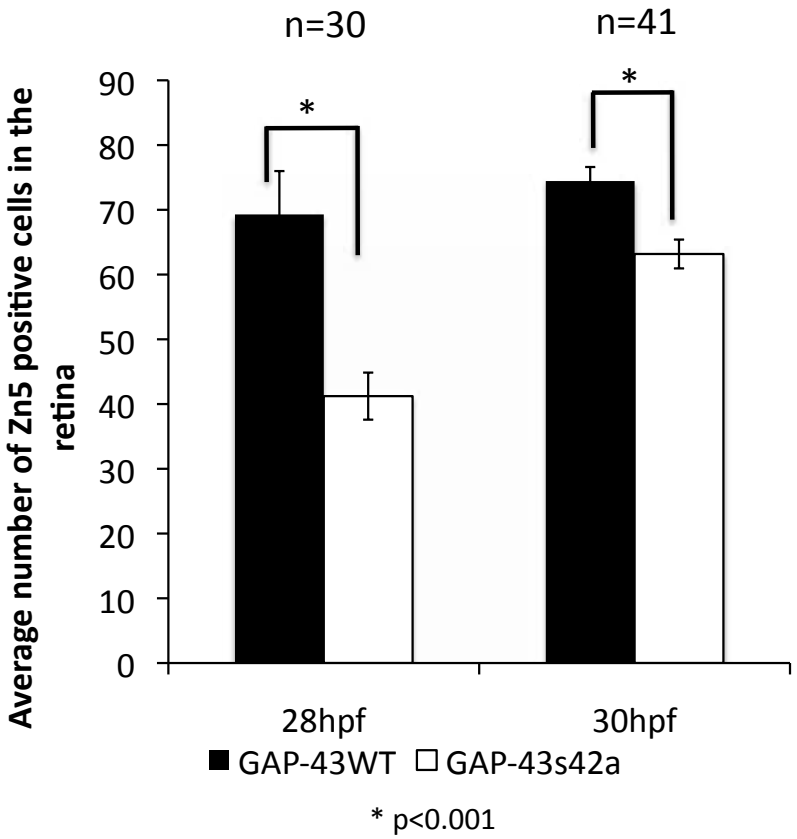

Figure 16. Fewer Zn-5 positive retinal ganglion cells in 48 hpf retinal sections of embryos overexpressing GAP43s42a starting at either 28 hpf or 30 hpf. The graph depicts the number of Zn-5 labeled retinal ganglion cells in a predetermined defined area of retinal sections expressing either GAP43s42a or GAP43WT (see page 30 and Fig. 2). GAP43s42a expression significantly reduced the number of RGCs in the retinal sections both at the 28 hpf and 30 hpf. In 28 hpf induced embryos GAP43s42a had an average of 41.2± 6.7 positive cells and GAP43WT had an average of 69.3± 3.6 positive cells ($p<0.001$). At 30 hpf GAP43s42a had 63.2± 2.2 and GAP43WT had 74.4± 2.2 ($p<0.001$). Images of retinal sections were magnified and analyzed using Metamorph®. Cells were counted in a 300μm^2 area of the retina bordered by the optic nerve and the lens. Significance was calculated using One-way ANOVA and error bars are standard error (28 hpf embryos n= 30 and 30 hpf embryos n=41).

A reduction in the RGC population could indicate the death of RGCs or a delay in the differentiation of neuronal precursors.

Reduction in the population of RGCs was caused by GAP43s42a overexpression delaying differentiation and increasing cell death

Previously, GAP-43 was shown to regulate asymmetric division of neuronal precursors in cerebellar granule cells (Gupta et al. 2010). We hypothesized that disruption of asymmetric division in GAP43s42-expressing retinal progenitors could lead to the observed reduction in the number differentiated RGCs and a concomitant increase in the number of dividing cells. Thus we used BrdU labeling of dividing cells to determine if the retinas of GAP43s42a overexpressing embryos had an increase in the number proliferating cells. We measured the number of dividing cells at 48 hpf, an age when the proliferation in the retina would be limited to proliferative zones at the margin of the retina. In comparison to embryos overexpressing GAP43WT, when GAP43s42a was induced at 28 hpf there was a large and significant increase in the number of cells labeled with BrdU at 48 hpf immediately following a one-hour pulse (Fig. 17B and 17C). When embryos overexpressed GAP43s42a starting at 30 hpf, there was also an increase in the number of cells labeled with BrdU, (when compared to GAP43WT), but this increase was not as large as the increase seen in 28 hpf induced embryos (Fig. 17D and Fig. 17E). GAP43s42a retinas induced at 28 hpf, had an average of 24.5± 2.0 BrdU positive cells and GAP43WT embryos induced at 28 hpf had an average of 2.2± 0.51 BrdU positive cells ($p<0.0001$). In GAP43s42a expressing embryos induced at 30 hpf, retinas had an

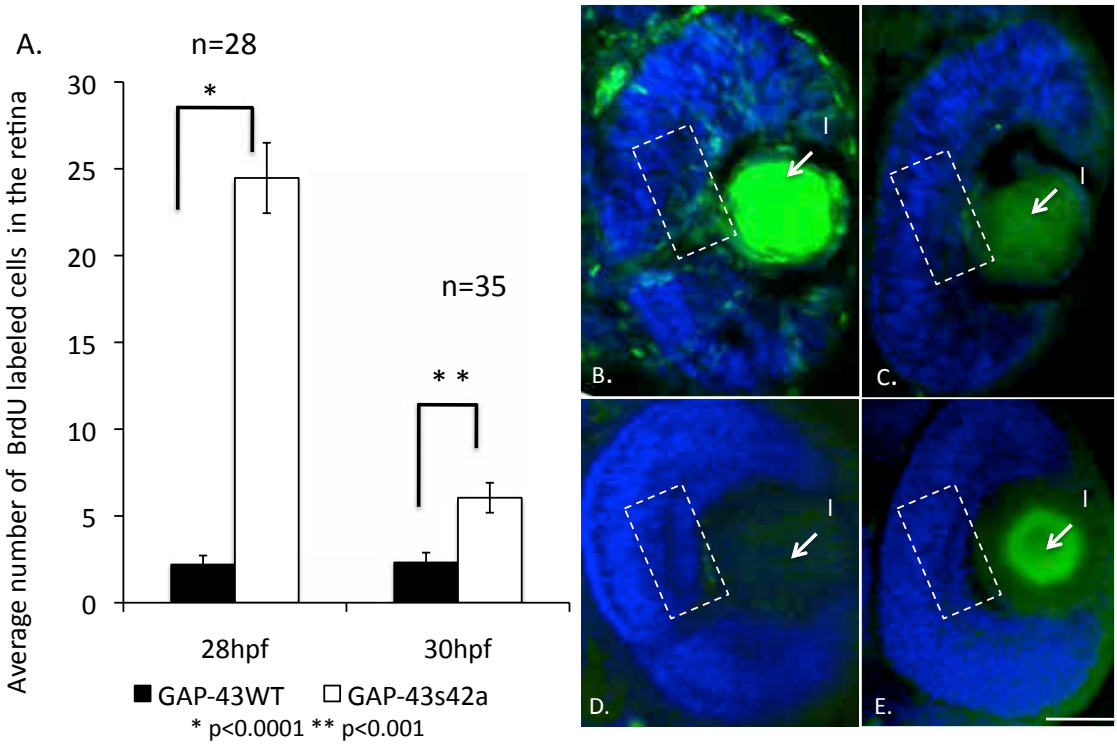

Figure 17. Increased BrdU labeled proliferative cells in the 48 hpf retinal sections of embryos overexpressing GAP43s42a starting at 28 hpf or 30 hpf. The graph shows the number of cells in a sample section of the retinal that are labeled with BrdU. The counts were conducted at 48 hpf in the retinas of embryos expressing either GAP43WT or GAP43s42a starting at 28 hpf or 30 hpf (A). When GAP43s42a was expressed starting at 28 hpf it caused a significant increase in the number of cell with proliferative capabilities (B) when compared to the number of cells labeled with BrdU in the GAP43WT 28 hpf retinas (BrdU green, DAPI blue (C)). In the retinas where GAP43s42a expression was induced at 30 hpf there was also a significant increase in the number of BrdU labeled cells (D) in comparison to retinas overexpressing GAP43WT starting at 30 hpf (E). The dashed square indicates the predetermined defined area where the counts occurred directly adjacent to the lens (white arrow l). In the 28 hpf induced embryos GAP43s42a retinas has an average of 24.5±2.0 positive cells and GAP43WT retinas had an average of 2.2±0.51 positive cells ($p<0.0001$). In the 30 hpf induced embryos GAP43s42a retinas had an average of 6.0±0.9 positive cells and GAP43WT retinas had an average of 2.3±0.6 positive cells ($p<0.001$). This increase was not as large as the increase in 28 hpf embryos. Cells were counted in a 300μm^2 area of the retina bordered by the optic nerve and the lens. Significance was calculated using One-way ANOVA and error bars are standard error (28 hpf embryos n=28 and 30 hpf embryos n=35). Fluorescent images were acquired, compiled, and processed with Metamorph® software. Scale bars =50μm.

average of 6.0± 0.9 BrdU positive cells and GAP43WT retina had an average of 2.3± 0.6 BrdU positive cells ($p<0.001$). When induction started at 30 hpf, there was agreement between the small increase in proliferative cells and the small decrease in the RGCs population.

A reduction in the number of RGCs could also be caused by RGCs undergoing apoptosis. To determine if cells in the retina of GAP43s42a overexpressing embryos were undergoing abnormal levels of apoptosis, we used an antibody for a marker of apoptosis, caspase 3, to probe retina sections. The average number of caspase 3 positive cells within the retina was greater in GAP43s42a overexpressing embryos than GAP43WT overexpressing embryos (28 hpf GAP43s42a retinas had an average of 3.2±0.3 caspase 3 positive cells, GAP43WT retinas had an average of 1.2± 0.4 caspase 3 positive cells ($p<0.01$), 30 hpf induced embryos GAP43s42a retinas had an average 5.7± 0.74 caspase 3 positive cells and GAP43WT retinas had an average of 3.5±0.8 caspase 3 positive cells ($p< 0.05$)). The increase in caspase 3 positive cells was apparent when GAP43s42a expression was induced at 28 hpf (Fig. 18B and 18C) and at 30 hpf (Fig. 18D and 18E). There was a significant increase in the number of cells undergoing apoptosis in the retinas of embryos overexpressing GAP43s42a, but levels of apoptosis were not enough to fully explain the reduction in the RGC population seen in the previous experiments. We also did not determine if the dying cells were Zn-5 positive RGCs, progenitors, or other cell types. In both GAP43WT and GAP43s42a retinal section there was fewer than 10 cells undergoing apoptosis in the retina (Fig. 18). A halt in retinal precursor differentiation resulting

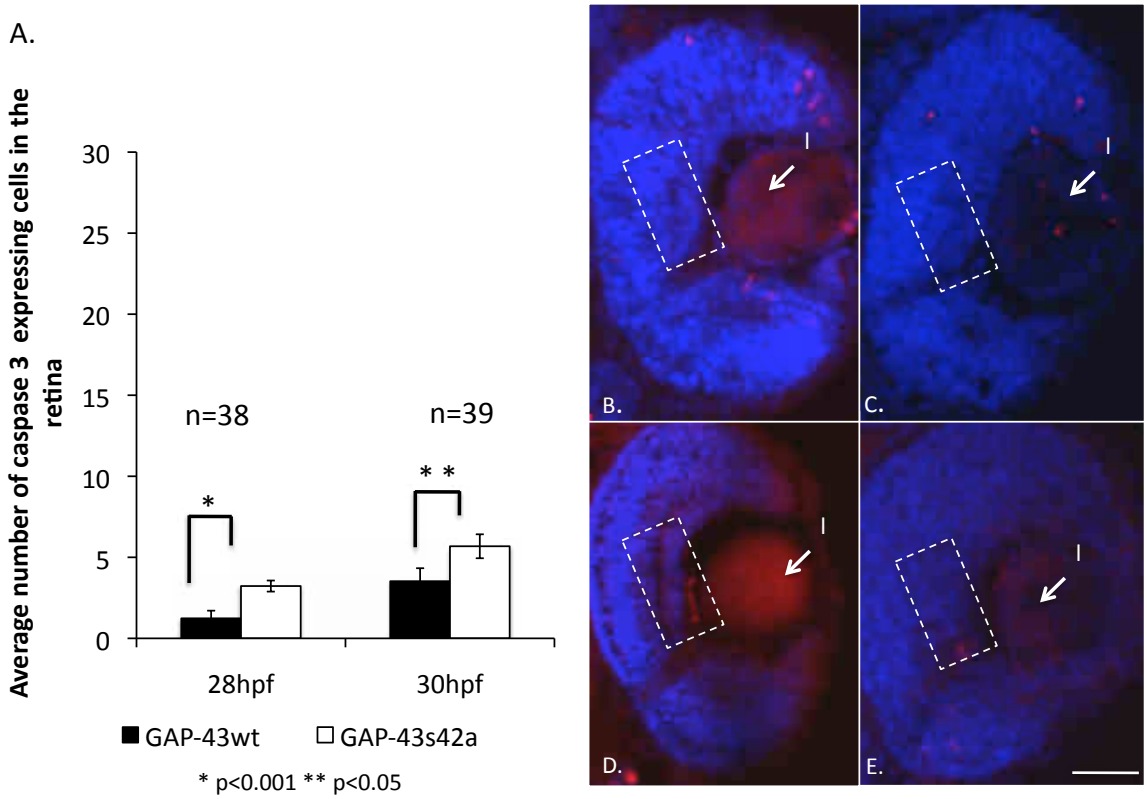

Figure 18. Increased caspase 3 expression in the 48 hpf retinal sections of GAP43s42a overexpressing embryos induced at 28 hpf or 30 hpf. The graph depicts the number of cells undergoing apoptosis (labeled with caspse 3 antibody) in a sample portion of retinal sections of GAP43s42a or GAP43WT overexpressing embryos (A). When GAP43S42A was overexpressed at 28 hpf (caspase 3 red, DAPI blue (B)) it increased the number of cells undergoing apoptosis in comparison to retinas overexpressing GAP43WT (C). Similarly when induction occurred at 30 hpf, GAP43s42a overexpression caused an increase in the number of cells undergoing apoptosis in the retina (D), when compared to embryos where GAP43WT was induced at 30 hpf (E). The dashed square indicates the predetermined defined area where the counts occurred directly adjacent to the lens (white arrow l). In the 28 hpf induced embryos GAP43s42a retinas had an average of 3.2±0.3 caspase 3 positive cells, GAP43WT retinas had an average of 1.2±0.4 caspase 3 positive cells ($p<0.001$). In the 30 hpf induced embryos GAP43s42a retinas had an average 5.7±0.74 caspase 3 positive cells and GAP43WT retinas had an average of 3.5±0.8 ($p< 0.05$). The increase in caspase 3 positive cells was slight but significant. Cells were counted in a 300μm^2 area of the retina bordered by the optic nerve and the lens. Significance was calculated using One-way ANOVA and error bars are standard error (28 hpf embryos n=38 and 30 hpf embryo n=39). Fluorescent images were acquired, compiled, and processed with Metamorph® software. Scale bars =50μm.

in a higher number of proliferative cells and the loss of some RGC could explain the partial reduction in the number of RGCs in GAP43s42a retinas.

Induction of GAP43s42a expression during early visual system development disrupts axon outgrowth in the retina

The reduction in the optic nerve diameter could also be explained by a failure of RGC axons to exit the retina. Guidance of axons within the retina including fasciculation into axon bundles is essential to exit the retina at the optic nerve head. Intraretinal guidance errors could contribute to the reduction of optic nerve diameter in embryos expressing GAP43s42a by reducing the number of axons exiting the retina. In order to address the pathfinding ability of axons within the retina, retinas probed with a neuronal marker (Zn-5) were flat mounted and the retinal cup was rotated to expose the lateral view of the RGC axons exiting at the optic disc. When induced to overexpress at either 28 hpf or 30 hpf, GAP43WT overexpressing retinas displayed an optic nerve with normal diameter exiting the retina (Fig. 19A and 19B). In contrast there was a reduction in the diameter of the optic nerve of the GAP43s42a overexpressing embryos induced at 28 hpf or 30hpf (Fig. 19C and 19D). This confirms that reduction in the diameter of the optic nerve was present directly after exiting the retina.

Within the retina, besides a reduction in the optic nerve diameter we also see evidence of guidance errors occurring as the RGCs axons attempt to navigate the OFL. To view the organization and fasciculation of RGC axons, the retina was flattened ventrally to obtain an overhead view of axons organization within the optic cup. Figure 20 displays the optic nerve organization in retinas from

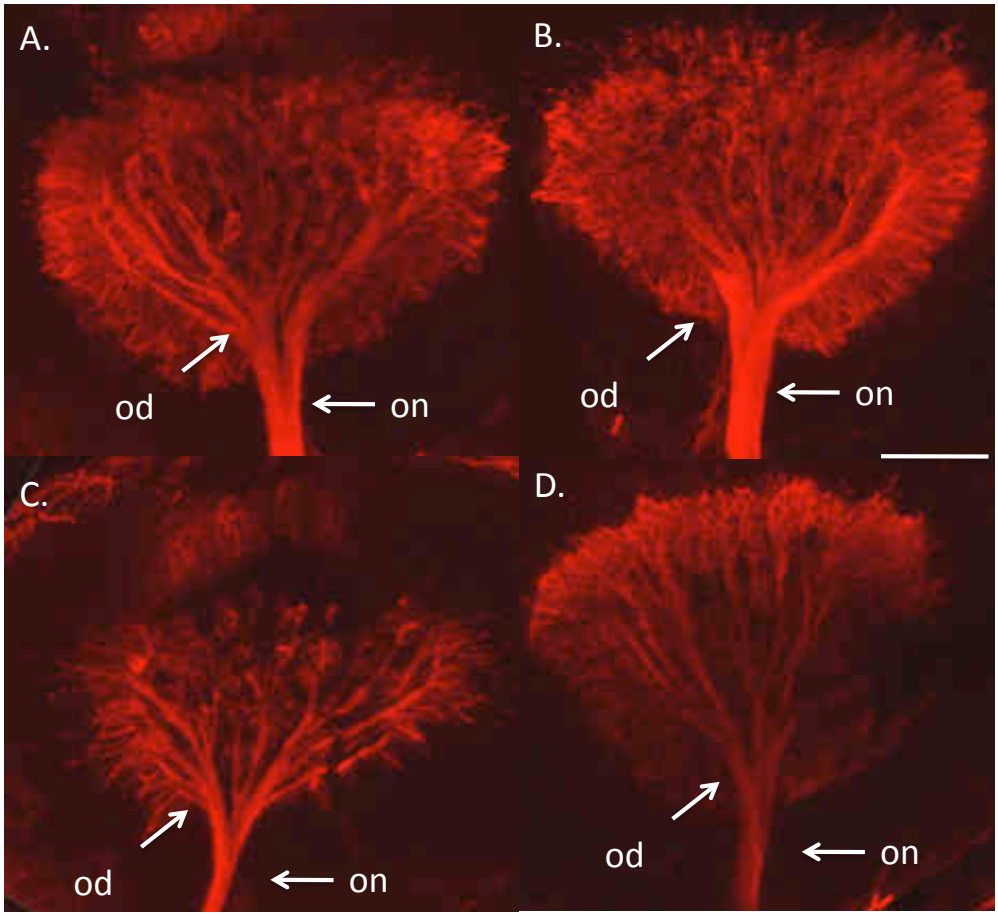

Figure 19. GAP43s42a overexpression starting at 28 hpf or 30 hpf reduces the optic nerve diameter at the optic disc in 48 hpf retinas. The retina was dissected from Zn-5 labeled embryos and imaged laterally to view RGC axons exiting the optic nerve disc. Images A and B are of embryos overexpressing GAP43WT with induction at 28 hpf and 30 hpf respectively. Images C and D are of embryos overexpressing GAP43s42a with induction at 28 hpf and 30 hpf respectively. When GAP43s42a overexpression was induced at 28 hpf it reduced the diameter of the optic nerve exiting at the optic nerve head (C) in comparison to the diameter of the optic nerve exiting the retina in GAP43WT overexpression embryos (induced at 28 hpf) (A). In comparison to the GAP43WT induced at 30 hpf, GAP43s42a induced at 30 hpf also displayed a reduced diameter of the optic nerve while exiting the retina (B and D). Images are of a flat mounted retinas where RGC axons have been stained with Zn-5 and rotated to display the lateral view of axons exiting the retina at the optic disc (od) and joining the optic nerve (on). Retinas were removed from the 48 hpf embryos and dorsally flat mounted in glycerol after the lens was removed. Confocal images were acquired, compiled, and processed with Metamorph® software. Scale bars =50μm.

GAP43s42a expressing embryos induced at either 28 hpf or 30 hpf. In the retinas of embryos overexpressing GAP43WT, numerous axons extend from the RGCs and guide towards the optic disc (Fig. 20A). In embryos induced at 28 hpf and 30 hpf to overexpress GAP43s42a, RGC axons appear disorganized and misguided along their pathway to the optic disc (Figure 20B and 20C white arrow). These results indicated that GAP-43 phosphorylation is required for guidance of RGC axons within the retina.

Upon higher magnification, axons leaving the ganglion cell layer in GAP43WT expressing embryos fasciculate with other axons creating dense tracts in the retina (Fig. 19D). The axon junctions occur early in the pathway towards the optic disc and fasciculation occurs across the majority of the GAP43WT axons length. In contrast in embryos overexpressing GAP43s42a, fasciculation of RGC axons was confined to lower portions of the axons and some axons failed to from dense bundles for the majority or the entirety of their length (Fig. 20E and 20F yellow chevron). When GAP43s42a overexpression was induced at either 28 hpf or 30 hpf it resulted in less dense bundles of axons in the retina. These results indicate that fasciculation is disrupted in embryos expressing GAP43s42a at 28 hpf and 30hpf, which could indicate the requirement of phosphorylated GAP-43 to properly respond to fasciculation cues. In both cases errors in fasciculation and guidance disrupted the organization of axons within the retina.

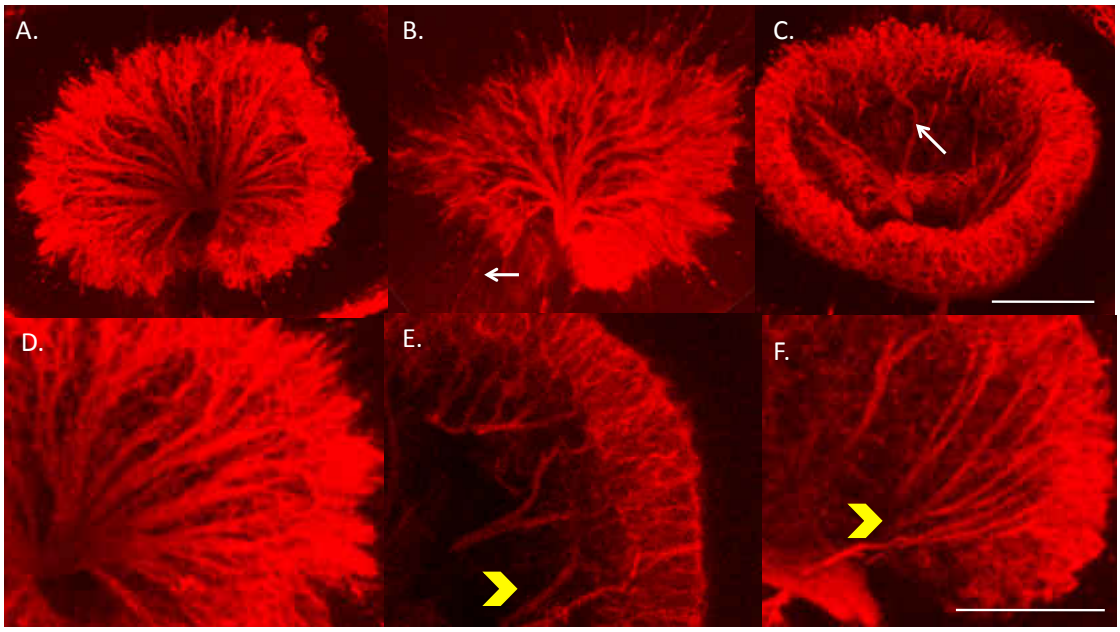

Figure 20. 48 hpf RGC axons overexpressing GAP43s42a display guidance and fasciculation errors in the retina. As previously mentioned the retina was dissected from Zn-5 labeled embryos and the RGC axons organization was compared between GAP43WT overexpressing embryos (A and D) and GAP43s42a overexpressing embryos (B, C, E, and F). Images B and E are of retinas from GAP43s42a overexpressing embryos induced at 28 hpf. Images C and F are of retinas from GAP43s42a overexpressing embryos induced at 30 hpf. Images A and D are of retinas from GAP43WT induced at 28 hpf (embryos induced at 30 hpf to express GAP43WT look identical). When GAP43s42 was overexpressed starting at 28 hpf or 30 hpf in the retina, it disrupted the guidance of RGC axons. Axons labeled with Zn-5 in the retina of GAP43s42a overexpressing embryos displayed guidance errors (B and C White arrows). In contrast, when GAP43WT was overexpressed in the retina the RGC axons displayed normal organization (A). Under higher magnification, the fasciculation of GAP43s42a overexpressing axons was disrupted (E and F) in comparison the fasciculation that occurs closer to cell body along the length of the GAP43WT expressing axons (D). Yellow chevrons indicate examples of defasciculated RGC axons. Retinas were removed from the 48 hpf embryos and flat mounted in glycerol after the lens was removed. The retinal cup was rotated to give a dorsal view of the axons bundles. Confocal images were acquired, compiled, and processed with Metamorph® software. Scale bars =50µm. Scale bar in C applies to images A-C and the scale bar in F applies to images D-F.

Inducing GAP43s42a late in visual system development resulted in a defasciculated optic nerve

Our previous experiments utilized an induction time targeted to perturb the GAP-43 phosphorylation during the differentiation of the RGCs and early axon guidance. Errors occurring when RGCs axons are within the retina could mask the effects of GAP43s42a overexpression on axon behavior in the optic chiasm. To address this question embryos were induced at 32 hpf, 34 hpf and 36 hpf and labeled with a neuronal marker (Zn-5) at 48 hpf to visualize axons in the GAP43s42a overexpressing embryos. Heatshocking at this time effects RGC axons as they are entering the optic chiasm and crossing to the contralateral optic tract. Similar to the result in the previous chapter, GAP43s42a overexpression during these stages in later development caused a defasciculated optic nerve phenotype. Figure 21 is representative of the defasciculation that only occurred when induction of GAP43s42a occurred after 30 hpf. The optic nerve is defasciculated particularly along the optic tract. In comparison, the optic nerve of GAP43WT overexpressing embryos contains densely compacted axon bundles. The defect was seen and quantified at each time point of induction. The increase in optic nerve diameter (at 32 hpf, 34 hpf and 36 hpf) was significant in comparison to the diameter of the optic nerve in control and GAP43WT overexpressing embryos that were heatshocked at any time including time points earlier than 30 hpf.

As expected, after induction at 32 hpf, embryos overexpressing GAP43s42a had a defasciculated optic nerve in comparison to the normally compacted optic nerve in GAP43WT overexpressing embryos (Fig 22A and 22B). To compare the

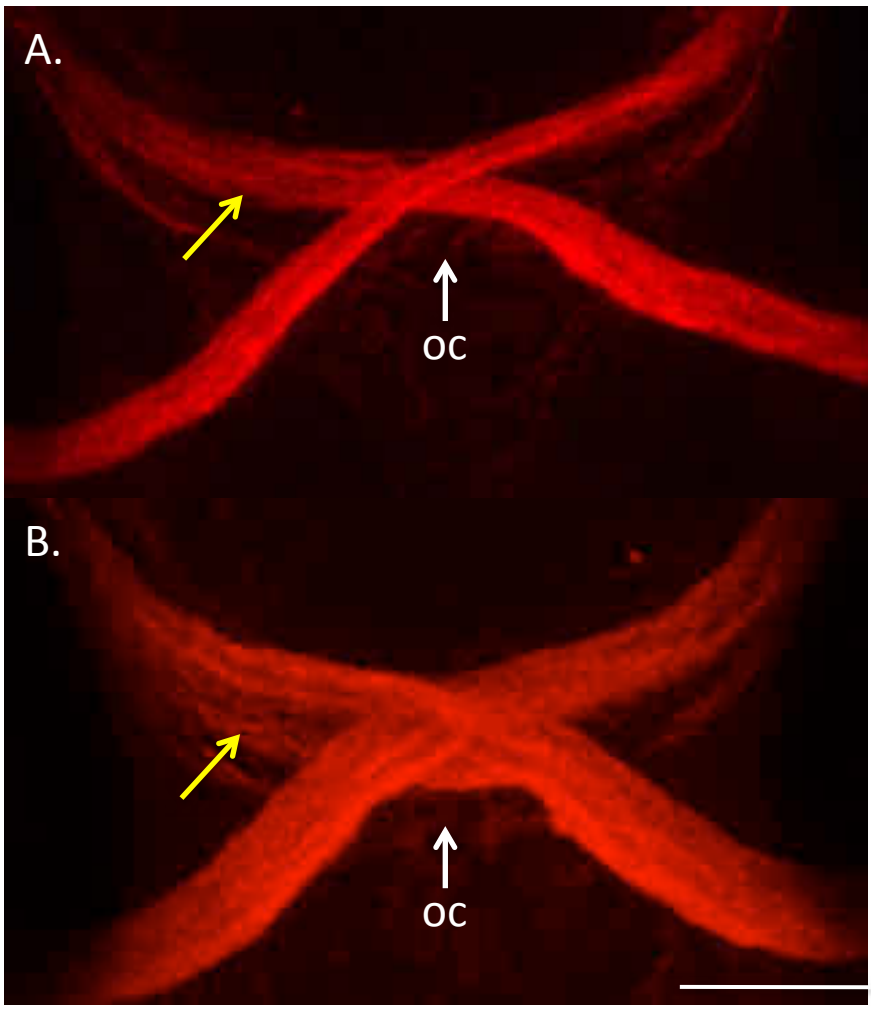

Figure 21. Overexpressing GAP43s42a during later RGC axon development caused a defasciculated optic nerve in 48 hpf embryos. Embryos were heatshocked at 34 hpf and then imaged with high magnification at 48 hpf to obtain a representative image of optic nerve defasciculation. GAP43WT expressing embryos displayed normal optic nerve diameter in the optic chiasm (A). When GAP43s42a was overexpressed it caused a defect in the fasciculation of the optic nerve (B). The yellow arrows indicate areas where axons of the optic nerve appear separated in embryos overexpressing GAP43s42a. In comparison the same area in embryos overexpressing GAP43WT appears normally compacted. Embryos were mounted ventrally to view the optic nerve within the optic chiasm (oc). Fluorescent images were acquired, compiled, and processed with Metamorph® software. Scale bars =50μm.

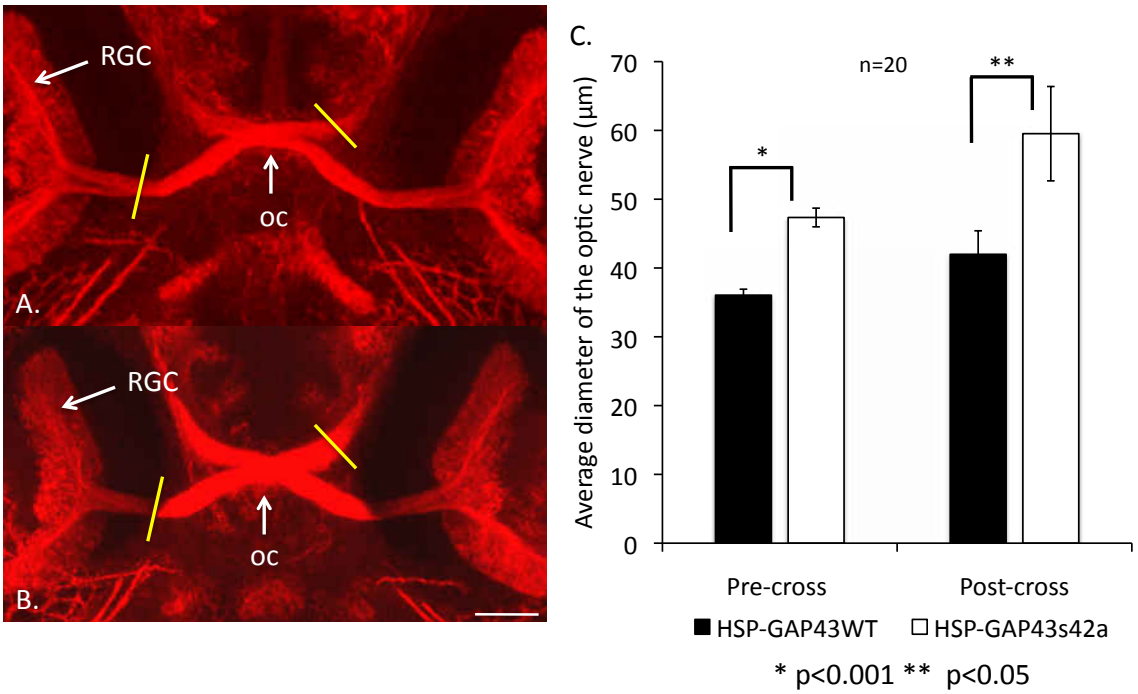

Figure 22. Overexpression of GAP43s42a induced at 32 hpf caused a defasciculated optic nerve in 48 hpf embryos. When GAP43s42a overexpression was induced at 32 hpf, it caused a defasciculated optic nerve (B) that appears wider in comparison to the optic nerve of embryos overexpressing GAP43WT starting 32 hpf (A). When the optic nerve was measured both before and after (yellow lines) the crossing at the optic chiasm (white arrows) there was a significant increase in the diameter of the optic nerve (C). Fixed and neuronal membrane labeled (Zn-5 antibody) embryos were mounted ventrally in glycerol for visualization and measurements of axons as they extend from retinal ganglion cell bodies (RGC) into the optic chiasm (oc). Confocal images were acquired, compiled, and processed with Metamorph® software. Scale bars =50µm. Error bars are standard error and these measurements were pooled from 4 replicates of the experiment (n= 20). Significance was calculated using One-way ANOVA.

increase in the diameter of the optic nerve, the width of the optic nerve was measured immediately after exiting the retina and after transecting the optic chiasm. After exiting the eye, the diameter of the optic nerve in embryos expressing GAP43s42a starting at 32 hpf was significantly increased when compared to embryos overexpressing GAP43WT starting at 32 hpf [GAP43s42a average diameter = 47.3±1.4 μm and GAP43WT average diameter = 36.1±0.9 μm] ($P<0.001$). The increase continues after the axons cross at the optic chiasm. When compared to embryos overexpressing GAP43WT, 32 hpf-induced embryos overexpressing GAP43s42a had a significantly wider optic nerve after transecting the optic chiasm [GAP43s42a average diameter = 59.6±6.9 μm and GAP43WT average diameter = 42.0±3.5 μm] ($p<0.05$).

When induction occurred at 34 hpf, the overexpression of GAP43s42a caused a similar increase in the diameter in the optic nerve in comparison to embryos overexpressing GAP43WT induced at the same time (Fig 23A and 23B). Again this reduction was quantified by the same two measurements, before and after the RGC axons cross in the optic chiasm. When overexpression was induced at 34 hpf the diameter of the optic nerve upon exiting the retina was significantly wider in GAP43s42a, embryos than those overexpressing GAP43WT [GAP43s42a average diameter = 48.9±1.6 μm and GAP43WT average diameter = 35.1±1.8 μm] ($p<0.05$). The GAP43s42a overexpressing embryos also displayed a defasciculated optic tract after crossing the optic chiasm, particularly when compared to GAP43WT optic nerves [GAP43s42a average diameter = 52.3±2.5 μm and the GAP43WT average diameter = 33.1±6.9 μm] ($P<0.001$).

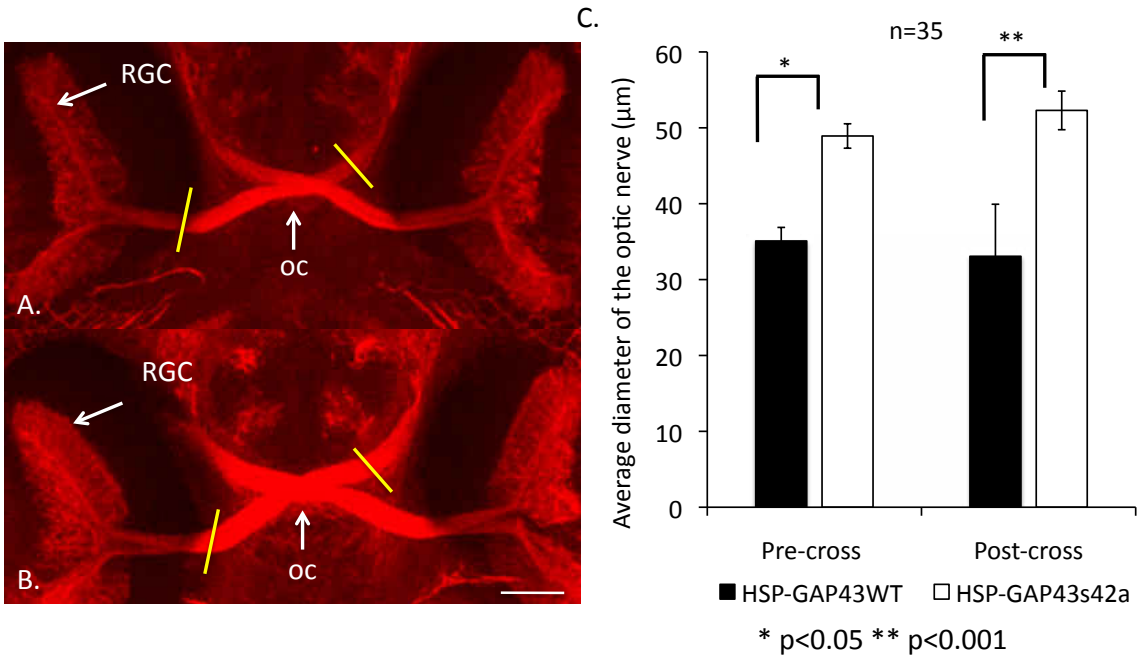

Figure 23. Overexpression of GAP43s42a induced at 34 hpf caused a defasciculated optic nerve in 48 hpf embryos. When GAP43s42a overexpression was induced at 34 hpf, it caused a defasciculated optic nerve (B) that appears wider in comparison to the optic nerve of embryos overexpressing GAP43WT starting 34 hpf (A). When the optic nerve was measured both before and after (yellow lines) transecting the optic chiasm (white arrows) there was a significant increase in the diameter of the optic nerve (C). Fixed and neuronal membrane labeled (Zn-5 antibody) embryos were mounted ventrally in glycerol for visualization and measurements of axons as they extend from retinal ganglion cell bodies (RGC) into the optic chiasm (oc). Confocal images were acquired, compiled, and processed with Metamorph® software. Scale bars =50µm. Error bars are standard error and these measurements were pooled from 4 replicates of the experiment (n= 35). Significance was calculated using One-way ANOVA.

At the latest induction time during RGC axon development (36 hpf), the overexpression of GAP43s42a also caused an increase in the diameter of the optic nerve in comparison to the diameter of the optic nerve in embryos overexpressing GAP43WT (Fig 24A and 24B). The increase was quantified using the same method mentioned above. Compared to the optic nerve of GAP43WT overexpressing embryos, the optic nerve of GAP43s42a overexpressing embryos was also significantly wider pre-optic cross when overexpression was induced at 36 hpf [GAP43s42a average diameter = 43.3±3.4 μm and GAP43WT average diameter = 36.2±1.5 μm] ($p<0.05$). The optic nerve was also wider after crossing the optic chiasm in embryos overexpressing GAP43s42a starting at 36 hpf [GAP43s42a average diameter = 46.9±2.8 μm and the diameter of GAP43WT = 38.8±2.0 μm] ($p<0.05$). These results indicate that GAP43s42a expression disrupts the fasciculation of axons within the optic nerve during the later stages of RGC axon development.

Guidance errors in the optic chiasm during double induction of GAP43s42a

When GAP43s42a overexpression was induced using heat at a single induction time (28 hpf-36 hpf), the RGC axons did not display any guidance errors in the optic chiasm. Previous research showed that fish injected with transgenes can have much higher levels of expression within a given cell than when the same construct is inherited as a stably integrated transgene. Thus we surmised that a higher level of GAP43s42a expression might be necessary to cause the major guidance errors we observed in the transient transgenic experiments. To determine

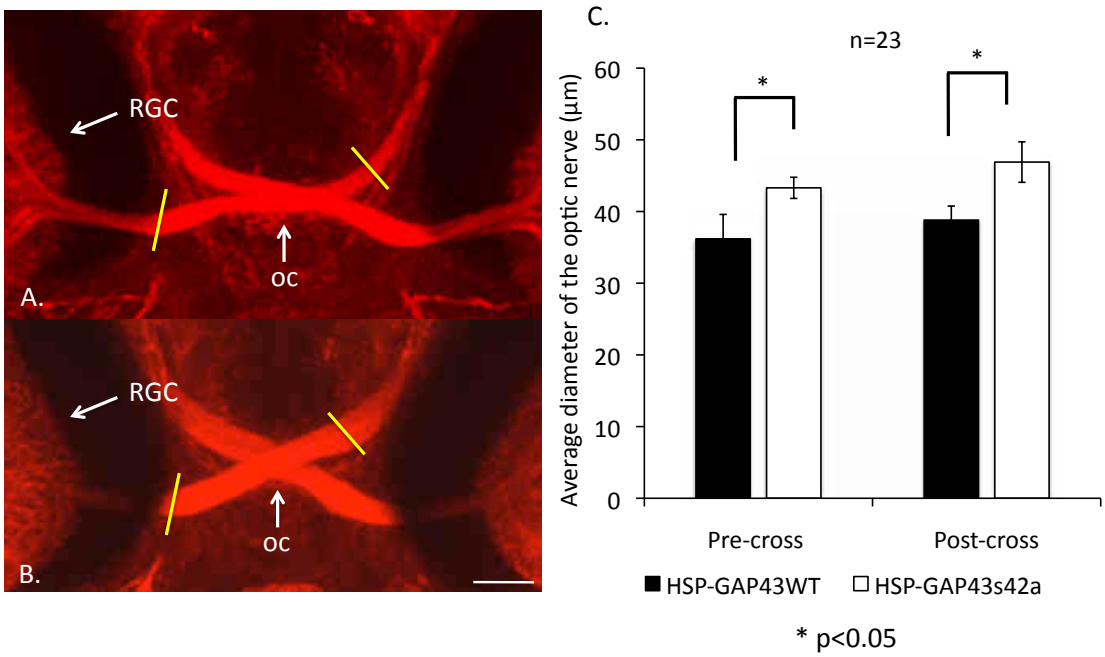

Figure 24. Overexpression of GAP43s42a induced at 36 hpf caused a defasciculated optic nerve in 48 hpf embryos. When GAP43s42a overexpression was induced at 36 hpf, it caused a defasciculated optic nerve with a wider diameter (B). It is wider in comparison to the optic nerve of embryos overexpressing GAP43WT starting 36 hpf (A). When the optic nerve was measured both before and after (yellow lines) transecting the optic chiasm (white arrows) there was a significant increase in the diameter of the optic nerve (C). Fixed and neuronal membrane labeled (Zn-5 antibody) embryos were mounted ventrally in glycerol for visualization and measurements of axons as they extend from retinal ganglion cell bodies (RGC) into the optic chiasm (oc). Confocal images were acquired, compiled, and processed with Metamorph® software. Scale bars =50µm. Error bars are standard error and these measurements were pooled from 4 replicates of the experiment (n= 23). Significance was calculated using One-way ANOVA.

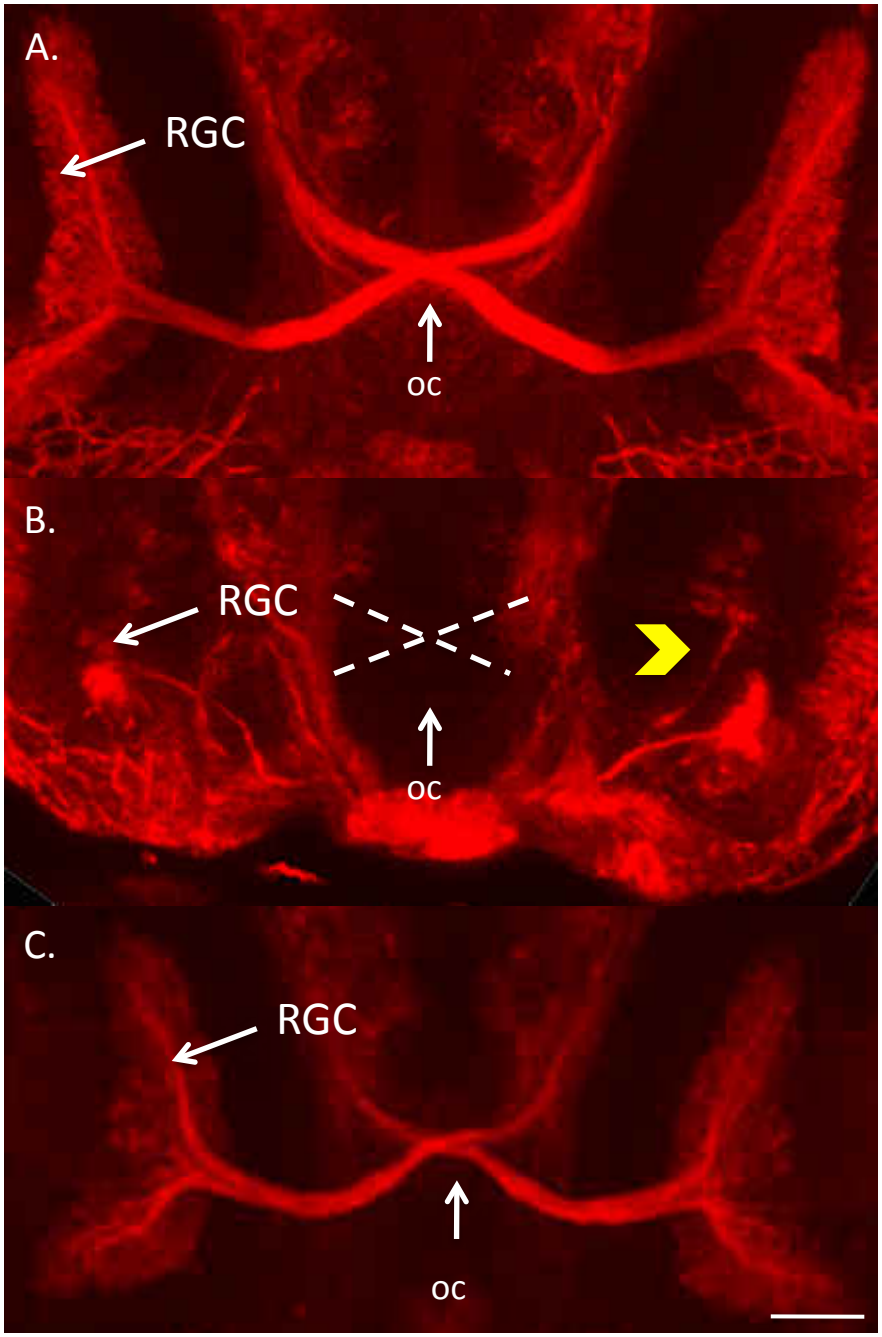
A.
RGC
OC
B.
RGC
OC
C.
RGC
OC

Figure 25. Embryos with double induction (31 hpf and 33 hpf) of GAP43s42a displayed guidance errors in the optic chiasm. Induction of these embryos was conducted twice, once at 31 hpf and again at 33 hpf. When GAP43WT expression was induced twice it resulted in normal pathfinding of the optic nerve (C) similar to the development of optic nerve in the non-heatshocked controls (A). In comparison, when GAP43s42a was induced twice during RGC axon development it resulted in axons growing in aberrant trajectories (B). The yellow chevron indicates axons that are taking aberrant pathways away from the optic nerve. The dashed X indicates where the optic nerve should cross the optic chiasm. Double induction indicates that robust expression of GAP43s42a was required to disrupt the regulation of the actin cytoskeleton of retinal ganglion cell (RGC) axons in response to guidance cues in the optic chiasm (oc). Fixed and neuronal membrane labeled (Zn-5 antibody) embryos were mounted ventrally in glycerol for visualization. Confocal images were acquired, compiled, and processed with Metamorph® software. Scale bars =50μm.

if a single induction of GAP43s42a overexpression was not meeting a threshold required to cause guidance errors, a double induction of the transgene was conducted to increase the expression of GAP43s42a. A heatshock was performed at both 31 hpf and 33 hpf to induce overexpression of GAP43s42a during times when RGC axons were actively pathfinding within the optic chiasm. In comparison to control (no heatshock) embryos and doubled heatshocked GAP43WT embryos (Fig. 25A and 25B), embryos overexpressing GAP43s42 at high levels (double heatshocked) displayed guidance errors in the optic chiasm (Fig. 25C). Again, similar to the transient results, the severity of the error varied in each embryo most likely due to differences in the response to heatshock.

Conclusions and summary

By limiting the expression of GAP43s42a to specific time points during RGC axon development, we were able to determine the independent roles of phosphorylated GAP-43 during RGC differentiation, axon fasciculation and guidance. From these experiments, we learned that phosphorylated GAP-43 is involved in the differentiation of RGCs and we hypothesize that competition between GAP43s42a

and endogenous GAP-43 for actin binding sites during RGC differentiation reduced the population of RGCs in the retina. Our experiments suggest that the loss of RGCs was due to halting differentiation of retinal precursors and we hypothesize that this was caused by disrupting axis orientation and organelle organization during the asymmetrical division of the precursors. Disrupting GAP-43 phosphorylation while axons are navigating the retina resulted in axon guidance defects that prevented RGC axons from exiting the retina. Inducing GAP43s42a expression after RGC axons leaving the retina disrupted the fasciculation of axon bundles in the optic nerve and the guidance of RGC axons in the optic chiasm. We hypothesize that in each case phosphorylation was required to regulate GAP-43's effect on the cytoskeleton in response to extracellular cues. The resulting defects were associated with distinct processes that required GAP-43 during different stages of RGC development.

Disrupting GAP-43 phosphorylation during the early development of RGCs in the retina disrupted the role GAP-43 has during RGC differentiation and intraretinal axon guidance. Inducing overexpression of GAP43s42a at 28 hpf and 30 hpf caused a significant and consistent reduction in the diameter of the optic nerve. Retinal cell counts and immunohistochemistry demonstrated that the reduction in optic diameter is associated with the loss of RGCs. Further probing of retinas overexpressing GAP43s42a with cell proliferation and cell death markers indicated that the reduction in RGCs is caused by a significant increase in the number of proliferative cells, and a slight but significant increase in apoptotic cells in the retina. We predict that GAP-43 phosphorylation is required for the previously described role of GAP-43 in the organization of cellular components during

differentiation and the acquisition of neuronal morphology (Strickler et al. 2006, Mishra et al. 2008). Disrupting the division of retinal precursors and loss of RGCs would decrease the number of axons comprising the optic nerve.

Flat mounted retinas from GAP43s42a overexpressing embryos also demonstrated that the reduction in optic nerve diameter is caused by guidance and fasciculation errors of RGC axons within the retina. When induction of GAP43s42a occurred at either 28 hpf or 30 hpf, axons within the retina appear disorganized leading to a possible failure to exit the retina. Guidance cues like slits, sema, NrCAM and LI within the retina are essential for the orientation of RGC axons towards the optic nerve head and the fasciculation of axons bundles (Thompson et al. 2006, Zelina et al. 2005, Oster et al. 2004). Our results mimic the inhibition of cues, like the membrane bound cell adhesion molecule NrCAM. When NrCAM was inhibited, it resulted in the failure of RGC axons to exit the retina (Zelina et al. 2005). We predict that the inability of RGC axons to regulate cytoskeletal dynamics when GAP43s42a was overexpressed rendered the RGC axons unable to properly respond to retinal guidance cues and exit the retina.

Disrupting GAP43 phosphorylation later in development highlighted the role GAP-43 has in the fasciculation and guidance of RGC axons in the optic chiasm. The overexpression targets a time when RGC axons have exited the retina, which disrupted the function of GAP-43 phosphorylation during axon guidance in the optic chiasm. When we induced GAP43s42a overexpression at 32 hpf, 34 hpf, or 36 hpf we disrupted the ability of RGC axons to respond to fasciculation cues that direct the compaction of axon bundles in the optic chiasm. Similar to the proposed mechanism

in the retina, GAP43s42a overexpression uncoupled fasciculation cues encountered by RGC axons from changes in the cytoskeleton required to compact the axons bundles. The defasciculated optic nerve was caused by disrupting signaling pathways important for fasciculation, like those involving NCAM binding, that result in GAP-43 phosphorylation (Meiri et al. 1998). Similarly guidance errors observed in the double heatshocked individuals overexpressing GAP43s42a were possibly due to a failure of RGC growth cones to respond to guidance and fasciculation cues. Again we propose that growth cone behavior was disrupted when communication was severed between molecules that direct changes in the cytoskeleton and guidance cues encountered by the growth cones of RGC axons.

GAP-43 phosphorylation is involved in the development of the visual system during multiple stages of RGC axonogensis. Prior to axon development, the phosphorylation of GAP-43 appears to be essential for the organization of the cytoskeleton during the final cell division leading to differentiation. Without phosphorylated GAP-43, the differentiation of RGCs is delayed; resulting in fewer axons in the optic nerve from RGCs. Disrupting GAP-43 phosphorylation also disrupts the intraretinal axon organization during development of the retina. GAP-43 phosphorylation was also disrupted in RGC after their axons have navigated the retina resulting in a defasciculation of the optic nerve and aberrant axon trajectories. GAP-43 phosphorylation is required for the progression of the axons from the initial stage of axon emergence to axonal pathway selection within the visual system of zebrafish.

CHAPTER V

Discussion

Using an inducible dominant negative mutation strategy to disrupt the closely regulated balance of phosphorylated and unphosphorylated GAP-43, we identified several critical roles for GAP-43 phosphorylation in the growth and guidance of developing retinal axons. Pan-neuronal expression of the dominant negative GAP-43 mutant, GAP43s42a, early in development resulted in a high mortality rate, the cause of which remains to be determined. However, induced overexpression of GAP43s42a later in development, when the retina is beginning to undergo neurogenesis, but after major CNS axonal tracts in the brain and spinal cord have been established, affected RGC differentiation and axon growth without impacting survival. GAP43s42a-induced defects included significant changes in optic nerve diameter, and more rarely, aberrant growth trajectories of RGC axons. The specific phenotypes varied based on the timing of GAP43s42a induction. Thus, the ability to regulate the timing of GAP43s42a induction with an inducible promoter allowed us to identify distinct RGC developmental processes requiring regulation of GAP-43 by phosphorylation.

In our system, GAP43s42a acts as a dominant negative due to induced overexpression during visual system development and endogenous expression in

neurons during early nervous system development. Robust expression of the phosphorylation mutant is key to overpowering the effects of endogenous GAP-43. Based on the results of *in vitro* actin-binding studies with the mammalian homologue of GAP43s42a, the observed effects of GAP43s42a overexpression on axon growth are likely caused by a competition for actin binding between the mutant and endogenous GAP-43 (Nguyen et al. 2009). Endogenous GAP-43 binds and stabilizes actin filaments when phosphorylated by PKC in response to external cues. Unphosphorylated GAP-43 has a lower affinity for actin, and acts to block actin polymerization. Since GAP-43s42a cannot be phosphorylated, it will block polymerization of actin filaments even in the presence of PKC activity. In our system, the overexpression of the mutant GAP-43 under the fG43 or HSP promoters would be expected to increase the amount of unphosphorylated GAP-43 available to block actin polymerization, unbalancing the ratio of phosphorylated GAP-43 to unphosphorylated GAP-43 in the growth cones, effectively quenching the activity of the endogenous phosphorylated GAP-43.

Disrupting GAP-43 phosphorylation during retinal neurogenesis and early axons growth in the retina reduced optic nerve width

During zebrafish retinal neurogenesis, progenitor cells differentiate to produce RGCs and other cells that compose the retina. Part of this process involves asymmetric cell division of the retinal progenitor, creating an additional progenitor and a post-mitotic cell or two post-mitotic cells with different fates (Poggi et al. 2005). RGCs are the first population of post-mitotic cells to emerge from retinal progenitors, and the fate of the post-mitotic cell is determined by the orientation of

the asymmetrical cell division (Insinna et al. 2010, Tgujkaw et al 2007). The cytoskeleton is regulated during this time to properly orientate the axis of division and to segregate cellular components between daughter cells. Using extracellular cues and intracellular molecules that direct cellular organization, the RGC cells develop a polarized morphology and a prominent neurite becomes the axon (Adler et al. 2006, Arimura and Kaibuchi 2007). The actin cytoskeleton is also responsible for providing structural support for the neurite that becomes the axon.

Using the inducible dominant negative GAP43s42a, we determined the role of GAP-43 phosphorylation during retinal neurogenesis and axon growth in the retina. Induced expression of GAP43s42a during differentiation of RGCs and intraretinal growth of RGC axons reduced the diameter of the optic nerve. Further experimental evidence indicated that this reduction in optic nerve diameter was caused by a reduction in the RGC population and disrupted intraretinal axon organization.

Disrupting GAP-43 phosphorylation delayed the division of retinal progenitors and reduced the population of RGCs

Overexpression of GAP43s42a significantly reduced the number of RGCs in the retina of stable transgenic zebrafish embryos at 48hpf. Embryos were induced to overexpress GAP-43 early during retinal neurogenesis, and prior to imaging treated with BrdU a thymidine analogue. BrdU is incorporated into the DNA of cells actively proliferating. DNA incorporation of BrdU and anti-BrdU antibody staining indicated an abnormally high number of proliferating cells in the retina of 48 hpf GAP43s42a expressing embryos. An abnormally high number of proliferative cells

and a reduction in the number of RGCs suggests that the presence of retinal progenitors unable to complete asymmetrical divisions that would produce the post-mitotic cells, one subgroup of which becomes the RGCs. A similar reduction and delay in differentiation was seen in other populations of cultured neurons and in the nervous system of heterozygous GAP-43 (+/-) knockout mice (McIlvain and McCasland 2006, Mani et al. 2001).

We hypothesize that expression of GAP-43s42a disrupted the regulation of the cytoskeleton halting the retinal precursors in a proliferative state by disrupting the asymmetrical division or organization of cellular components differentiating cells. RGCs are either prevented from completing asymmetrical division or from correctly segregating the cellular organelle that distinguished the daughter cells. *In vivo* and *in vitro* evidence demonstrated that phosphorylated GAP-43 is associated with axis orientation and organelle organization in differentiating neuronal precursors (Strickler et al. 2006, Mishra et al. 2008). Furthermore, cell culture experiments suggested that GAP-43 is essential for centrosome placement and spindle orientation in dividing neuronal precursors (Gupta et al. 2010). As mentioned earlier, the correct orientation of the centrosome and other cellular components determines the cell fate of post-mitotic cells and misoriented centrosomes halt asymmetrical cell division (Yamashita and Fuller 2008). Disrupting GAP-43 phosphorylation could disrupt the ability of cells to divide by causing the disorganization of centrosomes and spindles or disrupting other interactions between cellular organelles and the cytoskeleton. We concluded that disrupting the cellular organization of neuronal progenitors caused a delay in the

asymmetrical division, and within the retina this prevented retinal progenitors from producing RGCs. Consequently retinas of GAP43s42a overexpressing embryos retained proliferative progenitors unable to progress through neurogenesis and produce RGCs. Future experiments are needed to determine if there is a reduction in the number of other retinal neurons in embryo overexpressing GAP43s42a.

Neurogenesis in zebrafish initiates in the ventral retina and the wave of neurogenesis spreads to the nasal portion and finally to the dorsal and temporal portion of the retina (Neumann and Nuesslein-Volhard 2000, Biehlmaier et al., 2001). Because of the wave like progression of neurogenesis in the retina, there are population of retinal progenitors undergoing asymmetrical division, populations of progenitors that have yet to initiate asymmetrical division, and populations of differentiating post-mitotic cells all coexisting. A pulse induction of GAP43s42a could halt the progression of retinal progenitors that are actively dividing but would fail to delay the progression of those cells that have already committed to a RGC cell fate or have yet to asymmetrically divide. This window of sensitivity during neurogenesis would explain why there is only a partial reduction in the RGC population as opposed to a complete loss of RGC in GAP43s42a overexpressing embryos. The timing of induction also targeted different populations of cells during the wave of neurogenesis and therefore there was variation in the number of RGCs loss during the 28 hpf and the 30 hpf induction. Previous work has established that at 30 hpf, a portion of RGCs are already extending axons within the retina with the first few axons starting to reach the optic disc (Zhu et al. 1999). A larger portion of RGCs are differentiating at 28 hpf than at 30 hpf, reducing the amount of cells

affected during cell division by GAP-43s42a overexpression. Once these cells have committed to a RGC cell fate, our induction time could have already missed their asymmetrical division and would no long pause precursors in a proliferative state.

The ability of RGCs in embryos overexpressing GAP43s42a, to initially extend neurites was untested in these experiments, but impaired neurite extension could also contribute to the reduction in the optic nerve diameter. There is evidence in cultured cells that GAP-43 is required for neurons to extend neurites in response to attractive guidance cues like NGF, and NCAM (Meiri et al. 1998, Korshunnova et al. 2007, Mishra et al 2008). The inability of RGCs to extend axons could also originate from defects in organization of cellular components during differentiation. Similar to the regulation needed to organize the cytoskeleton during asymmetrical division, GAP-43 could be needed to organize the cytoskeleton and other components at the site of the future axon. When GAP-43 is depleted from cultured neuronal precursors, they fail to properly segregate and oriental the molecular components like the centrosomes, which are required to designate the position of axon extension (Mishra et al. 2008). Future experiments could use high-powered confocal time-lapse imaging of axons extension to determine if axon extension of RGCs is inhibited in embryos overexpressing GAP43s42a.

Apoptosis also contributed to the reduction in the RGC population in GAP43s42a overexpressing embryos. In zebrafish, some of the cells that stall during proliferation eventually undergo apoptosis (Plaster et al. 2006). While the number of cells undergoing apoptosis was significantly increased in GAP43s42a embryos, the number of cells undergoing apoptosis in our sample area was low. The low

levels of caspase 3 in the retina indicate that the delay in differentiation was not fatal to all stalled RGC progenitors at 48 hpf. As the embryo ages, perhaps a continued delay in differentiation would eventually cause apoptosis. To determine the extent of cell loss from retina as the embryos ages, future experiments would monitor any increase in apoptotic cells at later stages of development.

Disrupting GAP-43 phosphorylation disrupted the guidance and fasciculation of RGC axon in the visual system

Guidance of axons in the developing nervous system requires growth cones to obtain information about pathway selection from guidance molecules in the extracellular environment. But also essential is intracellular transduction of the information and modification of the cytoskeleton to produce the correct response to ligand binding. Overexpression of GAP43s42a disrupts the latter process by preventing the stabilization of the actin cytoskeleton in response to ligand binding and increased PKC activity. The ability to modulate the actin cytoskeleton in response to guidance molecules is disrupted and the resulting defect is determined by the temporal and spatial pattern of guidance molecules. Regulating the timing of induction therefore produces different defects dependent on the stage of RGC axon development. Disrupting GAP-43 phosphorylation when RGC axons are within the retina resulted in a disorganization of axons and reduced the diameter of optic nerve due to axons failing to exit the retina. Disrupting GAP-43 phosphorylation when RGC axons are within the optic chiasm resulted in the defasciculation of the optic nerve and aberrant axon trajectories.

Disrupting GAP-43 phosphorylation during axon guidance in the retina caused axons to take aberrant trajectories

Due to the wave like progression of differentiation and axon extension in the retina, the induction of GAP43s42a occurred during a time when a portion of the RGCs population was already extending axons into the OFL. Imaging of the retinal cup in GAP43s42a embryos, indicated that a reduction in the diameter of the optic nerve was also caused by a failure of RGC axons to exit the retina. RGC axons within the flat mounted retinas displayed both aberrant trajectories and defasciculation of axon bundles caused by disrupting GAP-43 phosphorylation.

Disrupting GAP-43 phosphorylation during RGC axon growth within the retina disrupted the ability of growth cones to correctly respond to guidance and fasciculation cues. In our experiments, GAP43s42a replaces endogenous GAP-43 during actin binding, disrupting the response to retinal guidance cues. Guidance cues including fasciculation cues within the retina are important for converging and exiting at the optic disc (Bao 2008). This includes slits, sema, NCAM and LI, which have been shown to be essential for the fasciculation and exit from the retina (Thompson et al. 2006, Zelina et al. 2005, Oster et al. 2003). Consequently, inhibition of cues, like the membrane bound cell adhesion molecule NrCAM, results in the failure of RGC axons exit from the retina (Zelina et al. 2005). Previous studies have established that GAP-43 phosphorylation is required for NCAM mediated growth (Meiri et al. 1998). When GAP-43 phosphorylation is disrupted, RGC axons are unable to respond to guidance cues correctly, and mimicking the loss of guidance molecules, fail to exit the retina.

Flat mounts of retinas from embryos overexpressing GAP43s42a also displayed an apparent reduction in diameter of the optic nerve at the optic nerve head. Axon bundles within the retina also appeared to have a reduced diameter and with fewer RGC axons fasciculating together. This further supports the hypothesis that the reduction in the diameter is caused by fewer RGC axons. This leads to the conclusion that the reduction in optic nerve diameter is caused by the combination of RGC axons unable to exit the retina and fewer RGCs.

GAP-43 phosphorylation is important for the growth and guidance of RGC axons entering the optic chiasm

Axons emerging from the retina encounter a pattern of guidance cues that direct the navigation and fasciculation of axons in the optic chiasm. Using the inducible promoter to regulate the timing of GAP43s42a expression, allowed us to determine the role of GAP-43 phosphorylation has during growth and guidance of RGC axons in the optic chiasm. Disrupting GAP-43 phosphorylation during the guidance of RGC axons within the optic chiasm resulted in the defasciculation of the optic nerve and aberrant axon trajectories in the optic nerve. The resulting defects in optic nerve fasciculation and axon guidance along the optic nerve, was specific to induction during this stage in RGC axons development.

Disrupting GAP-43 phosphorylation disrupted the fasciculation of axons bundles within the optic nerve

The wider optic nerve in the optic chiasm of embryos overexpressing GAP-43s42a was caused by defects in the compaction of axon bundles. The fasciculation of axons into bundles occurs over time as later emerging axons compact with those

that have already reached the chiasm. During the development of the retina the wave like progression of RGC differentiation and axon extension, causes axons to emerge from the retina in a staggered fashion. Initial axons that protrude into the optic chiasm are referred to as pioneer axons. Pioneer axons act as pathway indicators for the subsequent emergence of other RGC axons and facilitate the fasciculation of axon bundles into a tight nerve using adhesion molecules like L1 and NCAM (Walsh and Doherty, Weiland et al. 1997). It hypothesized that controlling the strength and frequency that L1 and other cell adhesion molecules bind either substrate or other cell adhesion molecules regulates axon guidance including guidance along pioneer axons. L1 is known to interact with the cytoskeleton via linker proteins (Wiencken-Barger et al. 2004). Actin filaments support the filopodia during axons guidance, which express cell adhesion molecules on their membrane. During fasciculation, GAP-43 could stabilize actin filaments that support the membranes containing cell adhesion molecules and therefore regulate the binding of cell adhesion molecules.

Disrupting GAP-43 phosphorylation and the regulation of the cytoskeleton removed the ability of RGC axons to respond correctly to the adhesion molecules on pioneer and other axons during fasciculation of the optic nerve. The explanation for this defect is supported by previous research documenting defasciculated nerves crossing the midline in the brain of GAP-43 (-/-) knockout mice (Shen et al. 2002). Disrupting the phosphorylation of GAP-43 produces similar defects because phosphorylation is the regulator of GAP-43 activity. The defasciculated nerve phenotype mimics the phenotype observed when adhesion molecules like L-1 are

depleted from the nervous system of zebrafish (Weiland et al. 1997). This supports the hypothesis that axons expressing GAP43s42a are unable to respond to adhesion molecules that direct fasciculation of the optic nerve.

NCAM is a possible candidate molecule involved in directing the fasciculation of the optic nerve through GAP-43 mediated stabilization of actin filaments. In support of this hypothesis, previous research indicated that GAP-43 is phosphorylated in response to NCAM binding (Korshunnova et al. 2007). As stated previously, the presence of GAP-43 was required for cultured cells to respond to NCAM signaling (Meiri et al. 1998). RGCs axons from embryos overexpressing GAP43s42a could fail to correctly respond to NCAM signaling by regulating the cytoskeleton and this could result in a defasciculated optic nerve.

Within the developing visual system, cell adhesion molecules are not the only fasciculation cues that direct the compaction of axon bundles. Previous research indicates the inhibitory guidance molecules help maintain tight fasciculation by preventing axon growth into aberrant territories. In mice, olfactory sensory neuron projections required inhibitory cues like sema3F to properly fasciculate en route to the accessory olfactory bulb (Cloutier et al. 2004). Consequently, defasciculation of the optic nerve could be caused by the removal of input from inhibitory cues. GAP43s42a expression acts to overwhelm the endogenous GAP-43 activity, and during the fasciculation of axons, upsetting the balance of phosphorylated and unphosphorylated GAP-43 could disrupt the modulation of growth cone morphology in response to inhibitory cues. Previous research has demonstrated the local collapse of inappropriate filopodia induced by inhibitory cues while the

remainder of the growth cone remains stabilized allows growth cones to smoothly turn away from a stimulus (Fan and Raper 1995). Overexpression of GAP43s42a could disrupt the delicate balance of unphosphorylated and phosphorylated GAP-43 during the smooth turn away from guidance cues. Future experiments are needed to determine if GAP-43 is involved in the response to any inhibitory cues within the optic chiasm.

Disrupting GAP-43 phosphorylation caused aberrant trajectories in RGC axons in the optic chiasm

In zebrafish, RGC axons emerging from the retina are guided across the midline at the optic chiasm, into the optic tract and towards their cellular targets in the tectum. Growth cones in the optic chiasm encounter guidance cues and collapse or extend filopodia towards those cues and these changes are achieved through the dynamic regulation of the cytoskeleton by proteins like GAP-43. As previously stated we hypothesize that the overexpression of GAP43s42a disrupts the ability of endogenous phosphorylated GAP-43 to regulate the cytoskeleton in response to ligand binding. When GAP-43 phosphorylation was disrupted during RGC axon navigation of the optic chiasm, it disrupted the guidance of axons within the optic nerve, particularly along the optic tract.

Our research and previous research demonstrated that GAP-43 and GAP-43 phosphorylation are required for the guidance of axons across the midline. GAP-43 knockout (-/-) mice show similar axon guidance errors across the midline and even a complete failure to form some axonal tracts across the midline (Kruger et al. 1998, Maier et al. 1999). Specifically in the visual system, GAP-43 phosphorylation

appears to be required to respond to guidance cues that direct axons across the chiasm and into the optic tract. When GAP-43 is depleted, RGC axons follow aberrant trajectories when trying to progress from optic chiasm to optic tract (Zhu et al. 1999). Previous research demonstrated that GAP-43 is required to respond to guidance signals from the lateral diencephalon. Axons of cultured RGCs required GAP-43 to respond to guidance signals from the lateral diencephalon cells (Zhang et al. 2013).

Previous research demonstrated that neither guidance cues nor fasciculation with pioneer axons is sufficient alone to direct axon tract formation. Correct axon pathfinding requires fasciculation and diffusible guidance cues. Therefore the defects in fasciculation caused by expression of GAP43s42a could be the source of aberrant trajectories in the optic nerve. Indeed, fasciculation with pioneer axons has previously been shown to facilitate the guidance of subsequent axons and removal of fasciculation caused guidance errors (Chitnis and Kuwada, 1991). In zebrafish, morpholino (small antisense interfering oligonucleotides) induced removal of pioneer RGC axons resulted in pathfinding and fasciculation errors of the later emerging RGC axons (Pittman et al. 2008). Therefore, it is possible that the disruption of GAP-43 phosphorylation was severe enough to inhibit the response of growth cones to fasciculation cues making the axons more susceptible to guidance errors. For example, if defasciculated axons project into ectopic territories that lack the local guidance cues or contain different guidance cues, growth cones will grow in aberrant trajectories. Fasciculation errors can be viewed as an initial less severe

misguidance of axons that in the right extracellular conditions could lead to more severe misguidance.

Axon guidance defects occurred less frequently than a defasciculated optic nerve

Compared to defasciculation of the optic nerve, which occurred frequently when GAP43s42a was overexpressed, axons growing in aberrant trajectories were rare in embryos overexpressing GAP43s42a. The relatively low frequency of axon guidance errors observed in these experiments could indicate that relatively strong overexpression of GAP43s42a was required to disrupt axons guidance. To overcome the regulation of actin by endogenous GAP-43 and other actin associated proteins, the induction of GAP43s42a must reach a significant threshold in individual axons. The high expression levels required to cause axons guidance errors could be indicative of either multiple signaling pathways or the redundancy of guidance cues/actin accessory proteins used during visual system development. In support of the hypothesis of multiple signaling pathways, previous research indicated that the zebrafish visual system employs multiple guidance cues including slits, semas and sonic hedgehog (Sakai and Holloran 2006, Neumann and Nuesslein-volhad 2000).

The increased number of GAP43s42a inductions required in stable transgenic embryos to cause RGC axon growth in aberrant trajectories, supports the hypothesis that disrupting axon guidance in the optic chiasm requires high expression of GAP43s42a. Injected transient transgenic embryos only required one heat induction of GAP43s42a to cause guidance errors, compared to the two

inductions of GAP43s42a required in stable transgenic embryos. The increased number of GAP43s42a transgene copies induced in the injected transient embryo, versus the stable transgenic embryos, could cause expression levels to more easily reach the threshold of GAP43s42a needed to cause guidance errors. In injected individuals the plasmid DNA remains extra-chromosomal through several cell divisions. The multiple copies are then able to insert into the genome at one or more sites. Stable transgenic embryos by comparison have only the number of insertions that occurred in the germ cells of the injected individual of the F0 generation. By selecting for individuals with germ line insertion, a population was created with the same number of insertions in the genome of every cell. The number of copies of the GAP43s42a transgenes in stably transfected cells is most likely lower than the number of copies present in an injected cell.

Increased mortality is associated with early disruption of GAP-43 phosphorylation

Pan-neuronal expression of GAP43s42a early in neurogenesis under the fG43 promoter caused a significant reduction in the survival of embryos from 24 hpf to 48 hpf. While the specific reason for the reduction in survival is unknown, results from the previous experiments in the developing visual system provide insight into how disrupting GAP-43 phosphorylation caused mortality. The defects in RGC differentiation, axon guidance, and fasciculation could reduce the vitality of younger embryos because these defects are occurring during a critical time period for nervous system development. Expression of GAP43s42a starting at 17 hpf under the *fugu* GAP-43-promoter, disrupts GAP-43 phosphorylation in the early stages of

zebrafish nervous system development and coincides with the formation of the basic anatomy of the nervous system including neural tube closure (Udvadia 2008, Papan and Campos-Ortega 1999).

During the early stage of nervous system development, stem cells within the neural tube give rise to two lineages, neuronal stem cells and glial stem cells, which in turn give rise to the many sublineages of neurons and glia within the nervous system. The continuous overexpression of GAP43s42a during widespread neurogenesis could delay or prevent the differentiation of multiple populations of neurons and extended delays in differentiation could eventually lead to apoptosis of progenitors. When GAP-43 was depleted from cultured neuronal precursors, it stalled differentiation and eventually leads to apoptosis (Mani et al. 2000). Mass apoptosis of neuronal progenitors in GAP43s42a overexpressing embryos would increase mortality. Future experiments would need to determine if there are abnormal levels of apoptosis in embryos injected with fG43-GAP43s42a-IRES-nucGFP starting earlier in development prior to the observed increase in mortality we observed at 24hpf.

As neurons of the developing CNS start to extend axons, axon guidance and fasciculation with pioneers are essential mechanisms used to establish axon connectivity. Axon connectivity during early development directly correlates with the nervous system function in the future. In zebrafish, guidance cues are important for directing early pioneer growth cones to establish the stereotypical axon tracts that act as scaffolds for later projecting growth cones to follow (Kuwada et al. 1990, Chitnis and Kuwada 1990). Our experiments in the visual system demonstrated that

GAP43 phosphorylation was required for RGC axon guidance and fasciculation of axons in the optic nerve. When GAP43s42a was overexpressed during the initial guidance of axons or the fasciculation of later emerging axons with pioneer axons, it could result in ectopic axon tracts or completely eliminate some axon tracts. Defects in connectivity could be severe enough to induce embryonic mortality. Future experiments are needed to determine if GAP43s42a expression during early nervous system development disrupts the formation of early axons tracts.

GAP43s42a expression could disrupt axons growth and guidance via an independent mechanism that facilitates actin disassociation

The results previously presented were interpreted based on the assumption that GAP43s42a is competing with endogenous GAP-43 for actin binding sites. We hypothesize that robust expression of the phosphorylation mutant GAP43s42a will out-compete the endogenous GAP-43 present in neurons. We have therefore until this point attributed the effects of GAP43s42a overexpression to the effects of removing phosphorylated GAP-43 activity during actin modification. Another possibility is that GAP43s42a has an independent role in growth cones during axons growth and guidance. GAP43s42a could not disrupt the activity of phosphorylated GAP-43 but instead operate through an independent mechanism to cause the defects previously discussed.

GAP43s42a could exert an effect on growth cone behavior by blocking actin polymerization. Previous research indicates that unphosphorylated GAP-43 could act as a "leaky" barbed end actin monomer capper, attenuating but not completely blocking actin polymerization. *In vitro* experiments revealed that incubation of

unphosphorylated GAP-43 with actin monomers increased the critical concentration required to polymerize actin monomers (He et al. 1997). It is therefore possible that the overexpression of GAP43s42a disrupted axon growth and guidance not by replacing phosphorylated GAP-43 on actin filaments but by binding actin monomers and attenuating actin polymerization.

Inappropriate blocking of actin polymerization via the overexpression of GAP43s42a could cause defects in neuron differentiation, axon guidance, and fasciculation of axons. For example during asymmetrical division abnormal amounts of GAP43s42a could block actin polymerization and prevent the cytoskeleton from moving organelles or centrosomes. During axons guidance and fasciculation abnormal amount of unphosphorylated GAP-43 could prevent the polymerization of actin filaments and the stabilization of filopodia in response to the binding of ligands to their receptors. It is possible that the defects observed in these experiments is the result of either GAP43s42a out competing endogenous phosphorylated GAP-43 for actin binding sites, GAP43s42a preventing the polymerization of actin monomers, or some combination of both activities. Future experiments are needed to determine the association of GAP43s42a with actin filaments or actin monomers.

Final summary

GAP-43, an actin accessory protein is enriched in neurons during differentiation and axon development. Phosphorylation of GAP-43 on serine 42 by PKC determines if GAP-43 has a stabilizing effect on actin polymerization or prevents the formation of actin filaments. By expressing a dominant negative phosphorylation mutant, we have indentified a role for GAP-43 phosphorylation in

the differentiation of RGCs, the guidance of RGC axons, and the fasciculation of RGC axons. During early visual system development, phosphorylated GAP-43 is required for retinal progenitors to differentiate into RGCs. Later in visual system development phosphorylated GAP-43 is required to couple the binding of guidance and fasciculation cues to the correct changes in the actin cytoskeleton. The results from these experiments offer insight into the complex interaction between growth cones and the extracellular guidance cues during the formation of the zebrafish visual system.

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- Graduate Student Travel Award. University of Wisconsin-Milwaukee. Travel award given to students to present at scientific conferences. 2008, 2009, 2010.

- McNair AOP Fellowship. Graduate Fellowship in Math and Sciences. 2006, 2007, 2008, 2009.

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c. Published article and selected presentations with published abstracts

- Angela Schmoldt, Jennifer Forecki, Dena Hammond, Ava Udvadia. Exploring differential gene expression in zebrafish to teach basic molecular biology skills. Zebrafish. Volume 6, Number 2, 2009

- Jennifer Forecki, Ava Udvadia. GAP-43 phosphorylation by PKC is essential for retinal ganglion cell development in zebrafish embryos. (*Manuscript in preparation)*

- **J. Forecki,** A.J. Udviadia. The role of GAP-43 phosphorylation in axons behavior in the developing zebrafish visual system. Poster Presentation. Aquatic Animal Models for Human Disease and Midwest Zebrafish conferences. Milwaukee WI. June 2013. Poster award recipient.

- **J. Forecki**, A. J. Udvadia. The role of GAP-43 phosphorylation during visual system development in the zebrafish. Society for Neuroscience Annual Meeting. Poster Presentation. New Orleans LA. October 2012.

- **J. Forecki**, A. J. Udvadia. GAP-43 phosphorylation and visual system development in zebrafish. Developmental Biology Gordon Conference. Poster Presentation. Andover NH. June 2011.

- **J. Forecki**, A. J. Udvadia GAP-43 phosphorylation and axon behavior in the developing visual system of transgenic zebrafish. Society for Neuroscience Annual Meeting. Poster Presentation. San Diego CA. November 2010.

- **J. Forecki**, A. J. Udvadia. Using transgenic zebrafish to understand the regulation of GAP-43 during axonogenesis in regenerating and developing neurons. Midwest Neuroscience Meeting. Poster Presentation. Milwaukee WI. April 2009. Best Graduate Student Poster Award.

- **J. Forecki**, A. J. Udvadia Effects of GAP-43 over-expression on axon guidance in the developing and regenerating visual system of transgenic zebrafish. Society for Neuroscience Annual Meeting. Poster Presentation Chicago Il. October 2009.

- Schmoldt, D.R. Hammond, **J. Forecki**, A. J. Udvadia, Genomic tools for identification of genes involved in neural development. Society for Developmental Biology Annual Meeting. Poster Presentation. University of Pennsylvania. Philadelphia. July 2008