Oncogenic Mutation of GNAQ/11 Disrupts Melanocyte Biology in a Zebrafish Model of Uveal Melanoma

by

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Submitted to the Department of Biology in Partial Fulfillment of the Requirements for the Degree of

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Abstract

Uveal melanoma (UM) is the most common primary intraocular cancer in humans. These tumors arise from nonclassical melanocytes found in the choroid, iris, and ciliary body (collectively called the uvea). Over 80% of UM tumors contain activating mutations in one of two genes encoding the homologous Ga proteins GNAQ or GNA11, which are the α subunit of a heterotrimeric G protein receptor. Here we report that we have successfully used Tol2-mediated transgenesis to develop a zebrafish model of UM driven by melanocyte specific expression of human oncogenic GNAQ/11\textsuperscript{209L} alleles. In cooperation with \textit{tp53\textsuperscript{M214K}} mutation, these GNAQ/11\textsuperscript{209L} transgenic zebrafish develop both uveal and cutaneous melanomas with nearly complete penetrance, causing early lethality. Furthermore, immunohistochemical analysis (IHC) reveals that these melanomas do indeed recapitulate the human disease in that they drive expression of oncogenic GNAQ and show increased expression of YAP. Interestingly, the transgenic zebrafish display an array of internal and external pigmentation defects compared to nontransgenic clutchmate controls. These pigment defects are independent of \textit{tp53} mutation. Zebrafish stripe patterning has been previously exploited to elucidate mechanisms in melanocyte signaling; therefore, to learn about the consequences of oncogenic GNAQ/11 signaling in melanocytes we pursued \textit{in vivo} and \textit{in vitro} study of transgenic melanocytes. The \textit{in vivo} pigment phenotypes in these mutants include hyperpigmentation, mislocalized melanocyte and aberrant melanocyte morphology, and are detectable through all developmental phases beginning as early as 3 days post fertilization. \textit{In vitro}, upon transgene expression the mutant melanocytes exhibit increased dendricity, motility, and survival. To understand the mechanisms underlying these phenotypes, we performed RNAsseq on transgenic and nontransgenic melanocyte populations. Transcriptome analysis revealed a list of 353 differentially expressed genes. Functional analysis of this gene set implied changes in categories related to axon guiding, cell adhesion and cell cycle regulation. Taken together, our results reveal that persistent alterations in melanocyte biology occur as a result of oncogenic GNAQ/11 expression in a novel zebrafish model of UM. These observations provide the ground work for elucidating the mechanisms by which the oncogenic GNAQ/11 mutations are tumorigenic and for potentially generating therapies for this poorly understood disease.

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# Table Of Contents

Abstract ........................................................................................................................................... 2  
Acknowledgements ......................................................................................................................... 3  

## Chapter One .............................................................................................................................. 7  
  
1.1. Chapter Overview ................................................................................................................... 8  
1.2. Introduction to Uveal Melanoma ............................................................................................ 9  
1.3. The Genetic Basis of Uveal Melanoma .................................................................................. 10  
  
1.3.1. Uveal melanoma initiating mutations GNAQ/11 ................................................................. 10  
1.3.2. Pathways downstream of oncogenic GNAQ/11 mutation in uveal melanoma .............................. 13  
1.3.3. Cooperating mutations in uveal melanoma ....................................................................... 16  
1.3.4. Animal models and prior clinical research ........................................................................ 18  
1.4. Introduction of Zebrafish as a Model Organism for Cancer ................................................. 21  
1.4.1. Introduction of zebrafish .................................................................................................... 21  
1.4.2. Brief history of cancer models in zebrafish ....................................................................... 22  
1.4.3. Modeling ocular disorders in zebrafish ............................................................................. 27  
1.4.4. Current tools and future directions for the study of cancer biology in zebrafish .............. 29  
1.5. Zebrafish Development and Stripe Patterning ...................................................................... 31  
1.5.1. Brief introduction to the study of pigmentation ................................................................. 31  
1.5.2. Overview of stripe patterning in zebrafish ....................................................................... 32  
1.5.3. Origins of the melanophore lineage and melanophore identity ........................................... 36  
1.5.4. Lessons learned from zebrafish pigmentation mutants .................................................. 39  
1.6. References .......................................................................................................................... 41  

## Chapter Two ............................................................................................................................. 52  
  
2.1. Abstract ................................................................................................................................. 54  
2.2. Introduction ......................................................................................................................... 55  
2.3. Results ................................................................................................................................. 58
2.3.1. Generation of a zebrafish model of uveal melanoma using Tol2-mediated transgenesis to express human oncogenic GNAQ behind a melanocyte-specific promotor........................................................................................................58
2.3.2. GNAQ/11Q209L oncogenic mutation cooperates with tp53 mutation in transgenic zebrafish to develop aggressive pigmented tumors .................................61
2.3.3. Melanomas in Tg(mitfa:GNAQ/11Q209L);tp53M214K/M214K zebrafish recapitulate human uveal melanoma YAP signaling ........................................65

2.4. Discussion........................................................................................................67
2.5. Methods ........................................................................................................70
2.5.1. Acknowledgements ..................................................................................74
2.6. Supplementary Figures and Tables .............................................................75
2.7. References ....................................................................................................77

Chapter Three............................................................................................................................80

Chapter 3: Table of Contents ...............................................................................................81
3.1. Abstract...........................................................................................................82
3.2. Introduction.....................................................................................................83
3.3. Results............................................................................................................87
3.3.1. Tg(mitfa:GNAQ/11Q209L) transgenic zebrafish display in vivo pigmentation defects throughout development .................................................................87
3.3.2. Background adaptation assays allow for quantitation of pigmentation defects in Tg(mitfa:GNAQQ209L) zebrafish.................................................................89
3.3.3. Tg(mitfa:GNAQQ209L) expression increases melanin distribution without increasing melanophore size.................................................................91
3.3.4. Tg(mitfa:GNAQQ209L) expression in melanophores results in increased dendricity and altered morphology.................................................................94
3.3.5. Tg(mitfa:GNAQQ209L) expressing melanophores have increased survival capacity ........................................................................................................98
3.3.6. Analysis of differential expression patterns of transgenic versus non-transgenic melanophore populations.................................................................100
3.4. Discussion.......................................................................................................110
3.5. Methods ........................................................................................................115
3.5.1. Acknowledgements ..................................................................................121
3.6. Supplementary Figures and Tables ...............................................................122
3.7. References ....................................................................................................132
Chapter Four

Chapter 4: Table of Contents ............................................................................................ 137
4.1. Overview ..................................................................................................................... 138
4.2. Ongoing and future experiments to interrogate the role of candidate genes of interest from RNAseq analysis in UM tumor progression ......................................................... 141
  4.2.1. Candidate genes of particular interest .................................................................. 142
  4.2.2. Ongoing and future experiments .......................................................................... 147
4.3. Conclusions and potential experiments utilizing phenotypic changes in transgene expressing zebrafish ........................................................................................................... 149
  4.3.1. Future experiments using zebrafish melanophore biology to elucidate the role of GNAQQ209L expression in signaling, proliferation and size .................................. 149
  4.3.2. Chemical screens utilizing Tg(mitfa:GNAQ/Q209L) embryonic phenotypes .......... 152
4.4. Ongoing efforts to improve our zebrafish model of UM ........................................... 152
4.5. References .................................................................................................................. 155
Chapter One

Introduction
## Chapter 1: Table of Contents

1.1. **Overview** .......................................................................................................................... 9

1.2. **Introduction to Uveal Melanoma** ..................................................................................... 9

1.3. **The Genetic Basis of Uveal Melanoma** ............................................................................. 10
   1.3.1. *Uveal Melanoma initiating mutations GNAQ/11* .................................................. 10
   1.3.2. *Pathways downstream of GNAQ/11 mutation in uveal melanoma* ............ 13
   1.3.3. *Cooperating mutations in uveal melanoma* ...................................................... 16
   1.3.4. *Animal models and prior clinical research* ...................................................... 18

1.4. **Introduction of Zebrafish as a Model Organism for Cancer** ........................................ 21
   1.4.1. *Introduction of zebrafish* ...................................................................................... 21
   1.4.2. *Brief history of cancer models in zebrafish* ....................................................... 22
   1.4.3. *Modeling ocular disorders in zebrafish* ............................................................... 27
   1.4.4. *Current tools and future directions for the study of cancer biology in zebrafish* ........................................................................................................................................................................... 29

1.5. **Zebrafish Development and Stripe Patterning** ................................................................. 31
   1.5.1. *Brief introduction to the study of pigmentation* .................................................. 31
   1.5.2. *Overview of stripe patterning in zebrafish* ......................................................... 32
   1.5.3. *Origins of the melanophore lineage and melanophore identity* ...................... 36
   1.5.4. *Lessons learned from zebrafish pigmentation mutants* .................................... 39

1.6. **References** .......................................................................................................................... 41
1.1. Chapter Overview

Uveal melanoma is a unique sub-category of melanoma arising from nonclassical melanocytes in the eye. Over 80% of uveal melanomas have mutations in homologous proteins GNAQ or GNA11, with specifically the \( GNAQ/11^{Q209L} \) mutation clearly outranking others as the most common point mutation. GNAQ/11 functions as the \( \Gamma a \) subunit of a heterotrimeric G-protein receptor complex upstream of a diverse set of signaling pathways. In this thesis, I will present a novel zebrafish model of uveal melanoma driven by melanocyte-specific oncogenic \( GNAQ/11^{Q209L} \) expression and the advancements in understanding the biology of \( GNAQ/11^{Q209L} \) signaling I achieved using this model. In this chapter, I will introduce uveal melanoma and discuss the reasons this malignancy has been thus far difficult to treat and study. I will introduce the history of zebrafish as a model organism for cancer, with a look to the future in development and usage of transgenic models. Lastly, I will introduce zebrafish stripe patterning and melanophore biology, and conclude with a discussion on how understanding the developmental pathways underlying stripe formation can help elucidate mechanisms in human disease.

1.2. Introduction to Uveal Melanoma

Uveal melanoma is the most common primary intraocular malignancy in adults, with tumors arising in around 2000 new cases each year in the United States alone, accounting for 3.7% of all melanomas (Jovanovic and Vlajkovic 2013). Uveal melanoma (UM) arises from the nonclassical melanocytes found in three structures of the eye, the choroid, iris and ciliary body, collectively called the uvea. The primary UM tumor is treatable with current technology; current methods of treatment include proton beam therapy, plaque radiation therapy or, most drastically,
enucleation surgery. While these treatments are effective, with the first two even preserving eyesight, UM remains a difficult disease to treat as within 15 years of primary diagnosis approximately 50% of patients present with metastases, almost entirely in the liver. The metastatic disease is essentially untreated, with median survival dropping to just 6 months after diagnosis (Shoushtari and Carvajal 2016).

Though our understanding of UM has progressed enormously in the past decade, many challenges remain in developing efficacious treatments. UM is genetically distinct from cutaneous melanoma, and therefore cutaneous melanoma treatments are not appropriate for UM patients. Standard chemotherapies have thus far proven ineffective, and further work is needed to develop targeted therapies (Shoushtari and Carvajal 2016). As UM is a rare disease, cell lines and patient samples are difficult to obtain and interpret; therefore, scientific advances using animal models are necessary in learning about and treating this malignancy.

1.3. The Genetic Basis of Uveal Melanoma

1.3.1. Uveal melanoma initiating mutations GNAQ/11

Studies in the past decade have finally begun to unravel the genetic and molecular mechanisms behind UM. In contrast with cutaneous melanoma, where BRAF or NRAS mutations are common, UM was found to be driven by oncogenic mutation in the α-subunit of a heterotrimeric G-protein receptor complex GNAQ. GNAQ mutations were first identified in UM in 2008 (Onken et al. 2008) and these finding were corroborated by a second paper in 2009 (Van Raamsdonk, Bezrookove, et al. 2009). Directly after this discovery it was reported that over
80% of UMls have mutations in either GNAQ or its homolog GNA11 solidifying the role of these two genes in UM initiation (Van Raamsdonk et al. 2010).

Like other proteins in the Gα family, GNAQ/11 have 2 domains: an α-helical domain creating a pocket for GDP binding and a Ras-like GTPase domain (Markby, Onrust, and Bourne 1993). G proteins function in signaling cascades by binding to a ligand-bound G-protein coupled receptor (GPCR) that catalyzes the release of GDP from the Gα subunit thereby allowing GTP binding. Once GTP-bound, conformational changes cause Gα proteins to dissociate from their Gβ and Gγ partners in order to then activate downstream signaling cascades (Figure 1.1A). Of particular importance to the disease is the Ras-like GTPase domain in GNAQ/11 that, through conformational changes, is responsible for the hydrolysis of GTP back to GDP to reset the signaling cascade. The two common GNAQ/11 mutations are in this Ras-like GTPase domain leading to an inability to hydrolyze GTP, thereby resulting in constitutive activation of downstream pathways (Van Raamsdonk et al. 2010; Van Raamsdonk, Bezrookove, et al. 2009). The two common mutations are a glutamine to leucine change at residue 209 (Q209L) or an arginine to glutamine or cysteine mutation (R183Q/C), with Q209L mutations found in ~90% of GNAQ/11 mutations (Cosmic database v69, Shoushtari and Carvajal 2014) and R183Q/C found about 4.8% of GNAQ/11 mutations (Van Raamsdonk et al. 2010) (Figure 1.1B).

The Van Raamsdonk group went on to try to address the functional relevance of a QNAQ versus GNA11 mutation and of Q209L versus R183Q/C mutation; however, if there are any differences they still remain unclear. While GNA11Q209L mutant melanocytes formed primary tumors faster than GNAQQ209L melanocytes when injected into mice (3-5 weeks vs 5-7 weeks), analysis of data from 81 human patients revealed no significant difference in disease-free
Figure 1.1. Schematic of GNAQ/11 mutations. (A) In the inactive GDP-bound state, GQ/11 is bound as part of a Gαβγ trimer. Upon ligand binding, its receptor catalyzes the exchange of GDP for GTP and GQ/11 dissociates from Gβγ to signal to its downstream effectors. In the absence of mutation, the Q209 and R183 residues would assist in the hydrolysis of GTP to GDP returning GQ/11 to its inactive state. (B) Schematic of GNAQ/11 protein structure highlighting the Q209 and R183 residues, located in the switch II and switch I regions respectively in the Ras-like GTPase domain required for GTP to GDP hydrolysis.
survival or overall survival (Van Raamsdonk et al. 2010). Furthermore, mice injected with immortalized cells expressing $GNAI1^{Q209}$ mutations developed more primary UM tumors than mice injected with cells expressing $GNAI1^{R183}$ mutations. Again, however, they report no difference in patient survival dependent on Q209L versus R183 mutation, suggesting that if functional differences exist in GNAQ versus GNA11 or Q209L versus R183Q/C mutation, those differences are not relevant to prognosis.

1.3.2. Pathways downstream of oncogenic GNAQ/11 mutation in uveal melanoma

Of the diverse downstream pathways activated in UM, the first and best understood signaling target of GNAQ/11 mutation is the mitogen-activated protein kinase (MAPK) pathway (Figure 1.2). Indeed, the first studies of primary and metastatic UM tumors found high levels of phosphorylated MEK and ERK in the absence of RAS or BRAF mutations prompting the search for the upstream effectors of MAPK pathway in this context (Zuidervaart et al. 2005). It had long been known that GNAQ/11 proteins signal through activation of phospholipase C beta-1 (PLC) (Lee et al. 1992). PLC can activate a number of downstream pathways, including MAPK, through the cleavage of phosphatidylinositol 4,5-bisphosphate (PIP2) into second messengers inositol triphosphate (IP3) and diacylglycreol (DAG) and subsequent activation of protein kinase C (PKC). PKC in turn leads to the activation of the MAPK pathway through sequential phosphorylation of RAF, MEK1/2 and ERK (Rozengurt 2007; Shoushtari and Carvajal 2014). GNAQ mutation was later proven to have a role upstream of phosphorylated ERK when immortalized melanocytes transfected with $GNAQ^{Q209L}$ increased expression of phosphorylated
ERK compared to those transfected with an empty vector (Van Raamsdonk, Bezrookove, et al. 2009).

GNAQ/11 signaling through PLC and PKC is not limited to MAPK activation. Interestingly, this pathway has been implicated in the mechanism of pigment translocation downstream of adrenergic signaling (Wu et al. 1992; Salim and A 2012). Second messenger IP3, generated as result of PIP2 hydrolysis, triggers mobilization of Ca\textsuperscript{2+} out of the endoplasmic reticulum (Rozengurt 2007). It has long been believed that pigment translocation downstream of hormonal and adrenergic signaling is dependent on intracellular Ca\textsuperscript{2+} influx (Vesely and Hadley 1979). Furthermore, Cos-7 cells transiently transfected with α-adrenergic receptors and either GNAQ or GNA11 were competent to release IP3 in response to adrenergic agonist norepinephrine compared to other Gα subunits (Wu et al. 1992). Cos-7 cells are not melanized, but taken together these experiments imply that if GNAQ/11 signaling released IP3 in a pigmented cell, the subsequent Ca\textsuperscript{2+} influx would trigger melanin translocation. Epinephrine and norepinephrine are well known agonists of melanin translocation in organisms, including teleosts (Logan, Burn, and Jackson 2006; Xu and Xie 2011). These results suggest a direct role of GNAQ/11 signaling downstream of adrenergic signaling and a suggested role in melanin translocation (Salim and A 2012)(Figure 1.2).

More recently, oncogenic GNAQ/GNA11 signaling has been shown to be partially mediated through YAP activation, which controls cell proliferation and tissue growth among other cellular processes (Field and Harbour 2014). Mutant G\textit{NAQ}/11\textit{Q209L} but not wild-type GNAQ/11 lead to de-phosphorylation and nuclear translocation of YAP independent of PLC signaling (Feng et al. 2014). Furthermore, there was a strong correlation in UM human patient
Figure 1.2. Schematic of selected signaling pathways downstream of wild-type and oncogenic GNAQ/11. GNAQ/11 signals to PLC which, through second messengers DAG and IP3, can eventually activate PKC triggering the MAPK cascade (left of figure) or can stimulate Ca\(^{2+}\) dependent pigment translocation (middle of figure). Via a PLC-independent pathway, oncogenic GNAQ/11 signaling through Trio can lead to Rho/Rac mediated F-actin polymerization which sequesters YAP-binding partner AMOT away from YAP. Free of AMOT, YAP may move to the nucleus to begin transcriptional programs mediating cell proliferation and tissue growth (right of figure).
samples between presence of GNAQ/11 mutations and YAP activation as measured by YAP nuclear translocation (Yu et al. 2014). Interestingly, this YAP activation is HIPPO-independent. A potential mechanism for YAP activation downstream of GNAQ/11Q209 was posited by Yu et al., who found that YAP activation required Trio, Rho and Rac1. Actin polymerization induced by Rho/Rac activation could sequester angiomotin (AMOT) from YAP, thereby freeing YAP to localize to the nucleus to activate transcriptional programs (Yu et al. 2014)(Figure 1.2). However, these mechanisms are not fully understood and more work is needed to understand the mechanism and role of YAP activation in UM. Importantly, these findings highlight that while GNAQ/11 signaling through PLC may be the most understood, it is certainly not the only, or necessarily the most important, mechanism by which oncogenic GNAQ/11 signaling promotes cancer. The discovery of GNAQ/11Q209L specific signaling in contrast to wild-type GNAQ/11 highlights our need for further understanding how UM signaling is being affected downstream of oncogenic GNAQ/11 mutation, and opens new possibilities in the field of potential therapeutics.

1.3.3. Cooperating mutations in uveal melanoma

As previously mentioned, about 50% of UM patients present with the incurable metastatic disease, therefore, research into cooperating mutations is necessary in establishing prognostic markers of metastasis and understanding how to treat the disease. It had long been appreciated that monosomy of chromosome 3 in UM was correlated with poor prognosis (Prescher et al. 1996), and in 2010 Harbour et al identified inactivating somatic mutations in the gene encoding BRCA1-associated protein (BAP1) in 84% of metastasizing UM tumors (Harbour et al. 2010).
BAP1 encodes a deubiquitinating enzyme that, among other functions, interacts with Additional Sex Combs like Transcriptional Regulator (ASXL1) to form the Polycomb group repressive deubiquitinase complex (PR-DUB) involved in regulation of homeobox genes and stem cell pluripotency (Scheuermann et al. 2010). Intriguingly, RNAi-mediated depletion of BAP1 did not result in increased proliferation, migration, invasion or tumorigenicity in UM cell lines and mouse xenograft injections (Matatall et al. 2013). However, siRNA depletion of BAP1 in UM cell lines led to loss of melanocytic phenotypes such as dendritic projections (Harbour et al. 2010) and RNAi depletion resulted in expression of stem cell related gene set enrichment pathways and enhanced ability to grow in stem cell conditions (Matatall et al. 2013). While future work will enhance our understanding of the molecular events downstream of BAP1 that result in poor UM prognosis, taken together these results demonstrate the importance of BAP1 inactivation in metastatic UM progression.

Other UM cooperating mutations have been discovered through gene expression profiling and transcriptome analysis and exome sequencing of primary UM tumors (Onken et al. 2004; DeBoever et al. 2015; Martin et al. 2013; Tschentscher et al. 2003). Expanding on work demonstrating the correlation between down-regulation and monosomy of chromosome 3 genes (Tschentscher et al. 2003), Onken et al went on to demonstrate a relationship between gene expression and patient survival. This type of gene expression analysis revealed that UM tumors cluster into two classes strongly correlated with prognosis for metastasis that discriminate low-grade (Class I) from high-grade (Class II) tumors (Onken et al. 2004). Genes that cluster Class I from Class II include significant clusters of down-regulated genes on chromosome 3 and up-regulated genes on chromosome 8q. Furthermore, down-regulation of as few as three genes, such
as PHLDA1 (pleckstrin homology like domain family A member 1), ENPP2 (ectonucleotide pyrophosphatase/phosphodiesterase 2) and FZD6 (frizzled class receptor 6), were sufficient to predict metastasis. In addition to these genes, exome sequencing revealed somatic mutation in splice factor SF3B1 and translation initiation factor EIF1AX that was almost exclusive of monosomy 3 (Martin et al. 2013). Both SF3B1 and EIF1AX have protective roles in UM metastasis, as patients with these mutations trend towards better prognosis (Ewens et al. 2014; Harbour et al. 2013). It is worth noting that the role of SF3B1 mutation appears to be context dependent and is not always protective in different cancers (DeBoever et al. 2015). In contrast to BAP1, SF3B1 is thought to act as an activating oncogene rather than a tumor suppressor as missense rather than nonsense mutations are frequently observed, and generally there is no loss of heterozygosity. While work has been done to attempt to elucidate the role of SF3B1 in UM and other cancers (DeBoever et al. 2015), these studies highlight the need to further research the role of these cooperating mutations to better understand their clinical importance.

1.3.4. Animal models and prior clinical research

As previously mentioned, approximately 50% of UM patients will eventually develop the metastatic disease. The prognosis for these patients is poor, highlighting the need for better UM models and clinical trials. Historically, UM clinical trials have involved cytotoxic treatments largely extrapolated from studies of cutaneous melanoma; now that we understand that cutaneous melanoma and UM have different genetic drivers it is unsurprising that these treatments have been ineffective (Shoushtari and Carvajal 2016). Insights into the mechanism of UM tumor signaling and growth have led to the first targeted therapeutic strategies in clinical trials. As most
patients with UM die of hepatic metastases, clinical trials typically focus on the hepatic metastatic tumors. A new wave of clinical trials attempted to use MEK inhibition, and demonstrated that selumetinib increased progression-free survival compared to other chemotherapies; however, the effect was modest with an increase from 7 to 16 weeks (Carvajal et al. 2014). Based on these results, clinical trials using MEK inhibition in combination with other therapies such as AEB071 for PKC inhibition are also underway (Shoushtari and Carvajal 2014). Yu et al applied verteporfin, a YAP inhibitor, UM cell lines mutated in GNAQ and BRAF and observed an increase in cell death in the UM cell lines with oncogenic GNAQ mutations whereas the BRAF-mutant lines appeared resistant (Yu et al. 2014). Verteporfin injection into an orthotopic mouse model of UM also inhibited tumor growth in vivo. These results present exciting new possibilities for future clinical strategies in UM treatment, however further investigation is needed to expand current therapeutic options as not one has shown any curative promise.

A good animal and/or cellular model is paramount in the study of any malignancy, and genetically modeling UM had not been possible prior to the discovery of the initiating GNAQ/11 mutations (Van Raamsdonk, Bezrookove, et al. 2009; Van Raamsdonk et al. 2010). Early attempts at modeling UM involved injection of a viral payload into eye tissue either in vivo or in vitro (Albert, Rabson, and Dalton 1968; Albert et al. 1981). The first attempt to model UM tumors, viral infection of cultured hamster eye tissue followed by subcutaneous injection for tumor formation, unfortunately yielded non-melanocytic tumors (Albert, Rabson, and Dalton 1968). The second attempt, viral injection directly into the anterior chamber of a feline eye, resulted in tumor cells with viral particles budding from the cell membrane (Albert et al. 1981).
The establishment of the first UM cell lines from both primary and metastatic tumors became an important and widely used tool (Luyten et al. 1996). Following the culturing of these cell lines, numerous studies focused on injection of human UM cells into the eyes of immunocompromised rabbits or mice (Hu et al. 1994; Yang et al. 2008; Mueller et al. 2002). Interestingly, cultured primary and metastatic UM lines were recently utilized in a zebrafish model to assay UM cell migration and proliferation (van der Ent et al. 2014). When injected into the yolk sac of 2-day old zebrafish, cell lines isolated from UM metastases proliferated and migrated more than cell lines isolated from primary tumors indicating that perhaps these enhanced proliferative and migratory capabilities are acquired during the metastatic process. Additionally, two experimental drugs added to the water of the engrafted embryos, quisinostat (a histone deacetylase inhibitor) and MLN-4924 (a neddylation pathway inhibitor), blocked migration and proliferation of two UM cell lines injected into zebrafish, demonstrating the potential utility of zebrafish experimentation in screening for UM inhibitors. Taken together, these studies moved forward the understanding of UM pathology, vascularization and metastases, but they are limited by the likelihood of acquired adaptive changes in cultured cells and the requirement of a suppressed or undeveloped immune system in the animal system.

Van Raamsdonk and colleagues had worked in mouse models involving GNAQ/11 mutation for more than a decade. Hypermorphic alleles of GNAQ/11 that increased dermal melanin were isolated as part of a screen to identify pigmentation mutants (Van Raamsdonk et al. 2004; Fitch et al. 2003). Phenotypic analysis demonstrated that GNAQ and GNA11 mutation were indistinguishable in this mouse model. Later in these same mice with hypermorphic GNAQ alleles (GNAQ/11^{Dsk}), they showed that GNAQ differentially regulates hair and skin
pigmentation dependent on localization in three separate compartments of the skin, the epidermis, dermis or skin (Van Raamsdonk, Barsh, et al. 2009). This experiment highlights that GNAQ/11 signaling in melanocytes can be context dependent. However, if these mice ever developed tumors it was not reported in these papers, and only recently have they introduced their first transgenic mouse model of UM (Huang, Urtatiz, and Van Raamsdonk 2015). In their mouse model of UM, Huang et al generated Rosa26-floxed stop-GNAQ<sup>Q209L</sup> mice crossed with Mitf-Cre mice to induce GNAQ<sup>Q209L</sup> expression in melanocytes (Huang, Urtatiz, and Van Raamsdonk 2015). Aside from a variety of interesting pigment phenotypes, these mice develop UM with short latency, usually within 3 months of age. Furthermore, these tumors overexpress YAP, further recapitulating the human disease. Murine models of UM such as this one represent a significant advance in generating tools in which to model the disease. Advances in this vein will hopefully lead to work increasing our understanding of oncogenic GNAQ<sup>Q209L</sup> signaling in the context of UM, but clearly further work is needed to translate the work of the UM scientific community to future clinically relevant therapies.

### 1.4. Introduction of Zebrafish as a Model Organism for Cancer

#### 1.4.1. Introduction of zebrafish

For decades, zebrafish have been recognized as a powerful tool in studying biology. Advantages for using the zebrafish as a model organism, particularly for the purpose of genetic studies, have been the subject of many reviews (Amsterdam and Hopkins 2006; Mione and Trede 2010; Berghmans, Jette, et al. 2005; White, Rose, and Zon 2013; Zhao, Huang, and Ye 2015). These advantages include high fecundity, transparency of embryos, ex utero development, small
size and relatively inexpensive maintenance. Importantly, zebrafish and humans share a high level of genetic and physiologic homology. Most mammalian organs have a zebrafish counterpart and approximately 70% of human disease genes have a functional homolog in zebrafish (Zhao, Huang, and Ye 2015; Santoriello and Zon 2012; White, Rose, and Zon 2013). Taken together, it is no surprise that the zebrafish has seen a huge gain in popularity as a model organism for human diseases, including cancer.

1.4.2. Brief history of cancer models in zebrafish

It had long been known that teleosts were capable of developing cancer, indeed the first melanoma model was reported in the 1920s in the related teleost Xiphophorus maculatus (Mione and Trede 2010; Meierjohann and Schartl 2006). The recognition that fish were also susceptible to neoplasia occurred as early as the 1960s (Stanton 1965). Around this time, zebrafish were mainly utilized as subjects in toxicology studies, but, in large part due to the efforts of Dr. Streisinger, began gaining traction as a powerful model for developmental biology in the 1980s. Even so, it was not until decades later that scientists truly began to appreciate the potential for zebrafish research in the field of cancer biology.

It was the realization that zebrafish were amenable to mutagen-based forward genetic screens that catapulted zebrafish into the field of developmental biology, and eventually opened the door for cancer modeling in zebrafish. Carcinogenic chemical treatments were developed for use in inducing tumorigenesis, the most common of which is N-ethyl-N-nitrosourea (ENU) (Beckwith et al. 2000; Grunwald et al. 1992) but other commonly used carcinogens were dimethylbenzathracene (DMBA) and N-methyl-N1-nitro-N-nitroguaniding (MNNG).
(Spitsbergen et al. 2000b, 2000a). Fish can develop a wide range of neoplasms in response to these carcinogenic treatments including various sarcomas and papillomas (Mione and Trede 2010).

As these large-scale mutagenesis screens gained traction, scientists began developing techniques to use these tools in a reverse genetics approach for understanding the consequences of genetic mutation of a specific gene. Following ENU-mutagenesis, a technique referred to as TILLING (targeting induced local lesions in genomes) became the most popular method for identifying mutations using a sequencing-based approach rather than identification by phenotype (Stemple 2004). Briefly, TILLING involved PCR amplification for an exon of interest in genomic DNA per fish in a mutagenized pool. The presence of point mutations in the genome can be detected either by direct sequencing of the products, or by melting and reannealing the PCR products causing a bulge in mutant strands upon reannealing that is then recognized and cleaved by an endonuclease. This cleavage event can then be detected on a sequencing gel.

Importantly, this kind of reverse genetic approach was used by Berghmans and colleagues to develop a zebrafish model to study the effects of a loss-of-function in the \( tp53 \) tumor suppressor gene (Berghmans, Murphey, et al. 2005). Zebrafish lines were generated from two different missense mutations in \( tp53 \) recovered following the kind of target-selected ENU-mutagenesis strategy described; and fish from one mutant line in particular, \( tp53^{M214K} \), spontaneously develop malignant peripheral nerve sheath tumors (MPNST). This mutation is located in the DNA-binding domain of \( tp53 \) and as such abrogates the ability of \( tp53 \) to bind to \( tp53 \)-specific consensus sequences in the DNA. The orthologous human codon for \( tp53^{M214K} \) mutation is mutated in 124 different human tumors and is located in a mutational hotspot of the
human TP53 gene (Berghmans, Murphey, et al. 2005; Olivier et al. 2002). Importantly, this study demonstrates that tumor suppressors, such as tp53, retain their evolutionarily conserved function between zebrafish and human.

In addition to generating mutants for disease models, transgenic expression of human or mouse oncogenes can also be a powerful tool to establish cancer models in zebrafish. Langenau and colleagues were the first to induce cancer by transgenic expression in zebrafish in the 2003 study expressing the mouse oncogene c-Myc under control of the rag2 (recombination activating gene 2) promoter (Langenau et al. 2003). Zebrafish expressing the zrag2-mMyc or zrag2-EGP-mMyc transgene rapidly presented with T-cell leukemia. Since this work, many transgenic cancer models have been developed in malignancies ranging from neuroblastoma, to AML, to melanoma (Table 1.1). To facilitate integration of transgenic genetic material in zebrafish genomes, new technologies arose such as the Tol2 transposition system. Tol2 contains an active transposase capable of catalyzing DNA transposition into zebrafish cells (Abe, Suster, and Kawakami 2011; Kawakami 2005). For integration in the zebrafish genome, donor plasmid containing DNA flanked by the Tol2 transposable elements together with mRNA encoding the transposase itself are injected into fertilized embryos. Tol2 facilitates good transgene expression and high integration frequency, thereby resulting in effective germline transmission, and marking this technology as a significant improvement over prior technologies (Amsterdam and Becker 2005).

A representative of a successful transgenic model cancer in zebrafish is the well-known melanoma model driven by oncogenic BRAF<sup>V600E</sup> expression behind the melanocyte specific promoter mitfa in cooperation with mutated tp53 (Patton et al. 2005). Though the melanoma
Table 1.1. Current transgenic zebrafish models available for the study of cancer.

<table>
<thead>
<tr>
<th>Cancer</th>
<th>Oncogene</th>
<th>Tumor suppressor?</th>
<th>Use in cancer biology</th>
<th>PMID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melanoma</td>
<td>mitfa-BRAFV600E</td>
<td>p53*</td>
<td>Genetic and chemical modifier screens</td>
<td>19694309</td>
</tr>
<tr>
<td></td>
<td>mitfa-EGFP:NRASG61K</td>
<td>p53*</td>
<td>Genetic modifier screens</td>
<td>19954345</td>
</tr>
<tr>
<td>Pancreatic</td>
<td>ptf1a-KRASG12V-GFP</td>
<td></td>
<td>Genetic modifier screens</td>
<td>21170325</td>
</tr>
<tr>
<td></td>
<td>ptf1a:Gal4-VP16 x UAS-KRASG12V-GFP</td>
<td></td>
<td>Cancer modeling, in vivo imaging</td>
<td>12574629</td>
</tr>
<tr>
<td>T-cell lymphoma/leukemia</td>
<td>rag2-myc</td>
<td></td>
<td>Genetic and chemical modifier screens</td>
<td>15627121</td>
</tr>
<tr>
<td></td>
<td><em>rag2-s lox-delRED2-lox-EGFP-mMyc x hsp70-cre</em></td>
<td>p53*</td>
<td>Inducible cancer models</td>
<td>17593023</td>
</tr>
<tr>
<td></td>
<td><em>rag2-NOTCH1</em></td>
<td></td>
<td>Notch1 interaction with bcl2</td>
<td>17252014</td>
</tr>
<tr>
<td>B-cell leukemia</td>
<td>Xenopus EF1a or zebrafish B actin — TEL-AML1 (ETV6-RUNX1)</td>
<td></td>
<td>Mechanisms of leukemia dissemination</td>
<td>17517628</td>
</tr>
<tr>
<td></td>
<td>b-actin-lox-EGFP-lox-KRASG12D x hsp70-cre</td>
<td></td>
<td>Inducible cancer models</td>
<td>20951945</td>
</tr>
<tr>
<td></td>
<td><em>krf4:Gal4VP16:14 x UAS:smoa1-EGFP x UAS:myrAKT1</em></td>
<td>p53*</td>
<td>Cooperation of hedgehog and Akt pathways</td>
<td>17018286</td>
</tr>
<tr>
<td>Rhabdomyosarcoma</td>
<td>rag2-KRASG12D</td>
<td></td>
<td>Identification of tumor initiating cell populations</td>
<td>17510286</td>
</tr>
<tr>
<td>Neuroblastoma</td>
<td>djh:EGFP-MYCN</td>
<td></td>
<td>Cooperation of MYCN and ALK</td>
<td>22439633</td>
</tr>
<tr>
<td>AML</td>
<td>djh:EGFP and djh:ALKF1174L</td>
<td></td>
<td>First model of AML in zebrafish</td>
<td>22439633</td>
</tr>
<tr>
<td>Lipoma</td>
<td>krt4Hsa.myrAkt1</td>
<td></td>
<td>Major tumor type found in p53-deficient zebrafish</td>
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</tr>
<tr>
<td>Ewing's sarcoma</td>
<td>hsp70 or beta actin-EWS/FLH</td>
<td>p53*</td>
<td>Platform for the study of drugs to treat lipoma and/or obesity</td>
<td>21979944</td>
</tr>
<tr>
<td>Liver</td>
<td>fabp10:LexPR; LexA-EGFP x crynCherry; LexA:EGFP-krasV12</td>
<td></td>
<td>Conserved function of EWS-FL11 fusion protein from human to fish</td>
<td>21903676</td>
</tr>
<tr>
<td></td>
<td>fabp10:TA; TRExmrk; krt4:GFP</td>
<td></td>
<td>Inducible KRAS hepatocellular cancer model</td>
<td>21888874</td>
</tr>
<tr>
<td>Pancreatic neuroendocrine</td>
<td>zmyod-MYCN</td>
<td></td>
<td>Inducible EGFR-homolog</td>
<td>15492244</td>
</tr>
<tr>
<td></td>
<td>Possible treatment of corticotroph tumors</td>
<td></td>
<td>Pancreatic neuroendocrine model as a platform for downstream MYCN targets NUP98-HOX9-induced oncogenesis from defects in haematopoiesis and aberrant DNA damage response</td>
<td>21810091</td>
</tr>
<tr>
<td>Myeloproliferative neoplasms</td>
<td>sp1(pu.1):NUP98-HOXA9</td>
<td></td>
<td>Identification of CDK inhibitors as possible treatment of corticotroph tumors</td>
<td>21538883</td>
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<tr>
<td>Corticotroph adenoma/ neoplasm</td>
<td>POMC-PPTQ (securin)</td>
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</tbody>
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Adapted from Richard A. White, 2015
model itself was not created using Tol2 technologies, Tol2 was later used to carry out genetic
screen in the Tg(mitfa:BRAFV600E); tp53M214K/M214K background. Melanoma is particularly
aggressive and among the more aneuploid cancers, and though genetic alterations such as
BRAFV600E and NRASQ16K were well documented it was generally believed that they could
cooperate with other somatic mutations during tumorigenesis. To this end, Ceol and colleagues
developed an approach to screen for modifiers of melanoma, and began their screen with 30
genes of interest from a region of recurrent chromosomal gain at 1q21 for genes that cooperated
with BRAFV600E driven melanoma (Ceol et al. 2011). To accomplish this, zebrafish expressing the
mitfa-BRAFV600E transgene in the context of a tp53 loss-of-function mutation were bred into the
nacre/mitfa mutant background, which due to mitfa mutation are devoid of melanocytes (Lister
et al. 1999). These animals will not develop melanoma, except in the event of melanocyte rescue
by the Tol2-mediated integration of a miniCOOPR plasmid encoding the mitfa gene. On the
same miniCOOPR plasmid a gene of interest from the 1q21 region may be included, thereby
ensuring that any melanomas arising in the transgenic fish contain both BRAFV600E and the gene
of interest. By this Tol2 mediated approach to screen BRAFV600E driven melanomas co-expressing
genes of interest on the miniCOOPR plasmid, Ceol et al discover that SETDB1, a histone
methyltransferase targeting a wide range of transcriptional targets, cooperates with BRAFV600E
expression to accelerate onset in melanoma. Genetic screens such as these demonstrate how
influential zebrafish can be as a platform for cancer research.
1.4.3. Modeling ocular disorders in zebrafish

The zebrafish eye is similar in morphology and physiology and function to the human eye (Santoriello and Zon 2012). Among other similarities to the human eye, the zebrafish eye includes melanocyte-containing anatomical structures such as the choroid and iris (Figure 1.3). Importantly, the zebrafish has been utilized in studying ocular development and disease. Optokinetic and optomotor behavioral studies of more than 400 zebrafish mutants uncovered mutants leading to lens degeneration (bumper), melanin deficiency (sandy) and optic nerve disorganization (grumpy and sleepy) among other ocular phenotypes (Neuhauss et al. 1999). Furthermore, pharmacological intervention in zebrafish mutants displaying eye defects and visual impairment have helped to elucidate the mechanisms of these diseases. For example, Lee et al used the zebrafish blowout mutant which harbors a mutation in patched1, a negative regulator of the sonic hedgehog pathway (Shh), to show that pharmacological inhibition of Shh pathway rescues the choroidal fissures common in the coloboma phenotype (Lee et al. 2008).

Several zebrafish models have also reported various cancers in the zebrafish eye. Interestingly, both Berghmans and Patton report eye tumors at low incidence in their MPNST and melanoma models respectively (Berghmans, Murphey, et al. 2005; Patton et al. 2005). It is worth noting that these cancers arose in different tissues in the eye, with tp53-driven MPNSTs commonly forming from the optic nerve tissue and BRAFV600E-driven melanomas forming in the choroid-associated melanocytes. Additionally, zebrafish mutants lacking functional copies of ptenb, one of two copies of the homolog for tumor suppressor gene PTEN, develop eye tumors in 7 months (Faucherre et al. 2008). These eye tumors were later diagnosed as hemangioarcomas by Choorapoikayil and colleagues, who then also went on to study pharmacological intervention of
the *pten* mutant phenotype by rescuing the embryonic mutant zebrafish with a PI3-kinase inhibitor (Choorapoikayil et al. 2012). These studies highlight how zebrafish models are functional and unique systems in which to model ocular diseases and malignancies.

**Figure 1.3. Histological section of the zebrafish eye.** Sectioning and subsequent H&E staining of the zebrafish eye (right panel) reveals conservation of human eye structures (schematic in left panel) such as the retinal pigmented epithelium (RPE), choroid and iris.
1.4.4. Current tools and future directions for the study of cancer biology in zebrafish

There is no question that there have been large advances in technology contributing to the benefits of using a zebrafish model in cancer research. So far described, the majority of transgenic zebrafish models are driven by overexpression of oncogenes. While chemical mutagenesis has allowed loss-of-function studies of putative tumor suppressors, such as \(tp53\), the increasingly popular CRISPR/Cas9 technology promises to emerge as a new technology to expedite engineering of precise genetic knockout zebrafish (Hwang et al. 2013). Several groups have already published on efficacy of CRISPR/Cas9 mutation methods for optimization (Burger et al. 2016; Shah et al. 2015). With this exciting new technology, large-scale reverse genetics for comprehensive testing and study of potential players in cancer now seems possible in zebrafish.

Another interesting technique and unique advantage to working in zebrafish is its amenability for \textit{in vivo} chemical screening. I previously described the use of chemical mutagens in zebrafish genetic screen; however, another chemical screening approach could involve using small molecules to uncover biological pathways or potential chemotherapeutics (White, Rose, and Zon 2013). Cancer-derived cell lines are frequently used to study the effects of antineoplastic drugs, however the lack of biological context in these screens commonly results in ineffectiveness of the drug when applied in an \textit{in vivo} setting. \textit{In vivo} vertebrate screens provide valuable information on anti-tumor effects, toxicity and pharmacokinetic data, however the cost of large-scale screens in mice is prohibitive. Zebrafish are ideal for these screens as these types of experiments can be done using embryonic fish with cancer-relevant phenotypes that can be used as proxies for efficacy. Screens can be efficiently carried out in embryonic fish as they are
optically clear, small, and easily generated through crosses. Embryos can be grown in a 96-well format and small molecule chemicals may be applied exogenously into the surrounding water or media manually or with liquid-handling machines.

An example of a successful screen carried out in embryonic fish was performed by White and colleagues, who screened around 2,000 chemicals for inhibitors of crestin+ lineage expression by in situ hybridization (White et al. 2011). They demonstrated up-regulation of neural crest genes crestin and sox10 upon transgene expression in the Tg(mifa:BRAF\textsuperscript{V600E};tp53\textsuperscript{M214K/M214K} melanoma model and reasoned that these programs were critical to melanoma growth. They discovered that inhibitors of metabolic enzyme dihydroorotate dehydrogenase (DHODH) abrogate expression of crestin, and that a chemical in the class of inhibitors, Leflunomide, does indeed inhibit melanoma growth in vitro and in mouse xenografts. The results of this screen led directly to clinical trials in human, and have hopefully opened the door for screens of this kind in other zebrafish cancer models.

Thus far I have discussed the evolution of zebrafish as a cancer model and have attempted to highlight some of the advantages of using zebrafish to model human malignancies. As the field moves forward, I believe it is necessary to find ways zebrafish can uniquely contribute to our knowledge of cancer in ways that are complementary to other mainstream models such as mice. In this section, I discussed technologies, such as genetic screens possible with advent of CRISPR/Cas9 and chemical screens, that take advantage of scalability of zebrafish and are thus far intractable in mice. Advantages such as these solidify the place of zebrafish as a resource in cancer research.
1.5. Zebrafish Development and Stripe Patterning

1.5.1. Brief introduction to the study of pigmentation

Studies in melanocyte biology and pigmentation date back over 100 years and have historically played a major role in the study of development and genetics. Indeed, in the first genetic experiments in mice, French biologist Lucien Cuenot used crosses of pigmented and albino mice to confirm Mendel’s laws in animals in 1902. Scientists today continue to study pigmentation and pigmentation mutants, as exemplified by the previously discussed large-scale mutagenesis screen in mice which revealed a class of phenodeviants with dark skin or hair. These mutants were described as darkskin (Dsk) mutants and were later discovered to be hypermorphic alleles in GNAQ/11 (Hrabé de Angelis et al. 2000; Fitch et al. 2003; Van Raamsdonk et al. 2004).

Melanocytes and their progenitor cells, melanoblasts, provide an excellent model for development. They represent a cell lineage that differentiates from a multipotent stem cell, they migrate through the developing animal interacting with their environment as they move to localize to a specific site in the organism, and they generate a stem cell population from which they self-renew. It is perhaps these properties of melanocytes and melanoblasts that contribute to the aggressive and metastatic nature of melanoma. Our continuing efforts to enhance our knowledge of melanocyte biology have, and will continue to, contribute to our understanding of melanoma and inform the design for new treatments strategies of this malignancy.

In this section, I will discuss melanocytes (called melanophores in zebrafish) and their progenitors more thoroughly in the context of zebrafish. I will also illustrate cross-species
similarities between mammalian and zebrafish pigmentation as a rationale for why pigmentation studies in zebrafish can contribute to our knowledge of melanocytes and their malignancies.

1.5.2. Overview of stripe patterning in zebrafish

The adult zebrafish pattern is characterized by alternating patterns of four or five dark stripes and four light stripes covering the body of the fish as well as the anal and tail fins (Figure 1.4A). Fascinated by the question of how these stripes and other beautiful and complex patterns formed in nature, Alan Turing famously developed a mathematical biological model in 1952 to describe the activity at a molecular level that could give rise to such patterns (Turing 1952). His model of a pattern formation mechanism called “reaction-diffusion” has largely held up over the course of 60 years (Kondo and Miura 2010). The reaction diffusion (RD) model describes how patterns can form as a consequence of two diffusible elements interacting with each other while diffusing at different rates. These two elements are a locally self-enhanced element which activates the other element that acts as its long range inhibitor (Kondo and Miura 2010; Metz, Manceau, and Hoekstra 2011; Watanabe and Kondo 2015). Indeed, zebrafish pigmentation fulfills both the local activation as well as the long-range inhibition dynamic requirement of the RD model. Of course the next challenge is to determine the identity of the determinants of the in vivo molecules.

Though the RD model only requires 2 diffusible elements, zebrafish pigmentation patterns arise from interaction among three types of pigment cells, or chromatophores. These three players in the zebrafish stripe pattern are the melanophore, xanthophore and iridophore. The melanin-containing melanophore is the cell homologous to the mammalian melanocyte,
indeed even in fish at times “melanophore” and “melanocyte” are used interchangeably.

Xanthophores are characterized by a yellow-orange color that results from their synthesis of a pteridine pigment. Lastly, iridophores are filled with light-reflecting guanidine platelets and are responsible for the silvery appearance of many fish. The chromatophores share a common progenitor, namely neural crest cells, which also give rise to neural tissue (Figure 1.4B)(Le Douarin and Dupin 2003). The short-range repulsion and long-range survival cues from the

Figure 1.4. Overview of zebrafish stripe patterning. (A) The stripe pattern in the adult zebrafish is characterized by an alternating stripe/interstripe (dark purple arrow/yellow arrow) pattern across the body of the fish extending into the anal and tail fins. (B) The 3 chromatophores responsible for the stripe pattern, melanophores, iridophores, and xanthophores, share neural crest progenitors with each other and neuronal cells.
xanthophore to the melanophore drive stripe formation, but the specific contribution of iridiphores to this patterning is less well understood (Singh and Nusslein-Volhard 2015).

Though the exact molecular mechanisms of stripe formation are not yet fully known, the sequence of stripe formation has been characterized throughout development in zebrafish (Kelsh et al. 2009; Parichy and Spiewak 2015; Singh and Nusslein-Volhard 2015). Zebrafish development, and patterning, can be divided into three stages: embryonic, metamorphic and adult (Figure 1.5). The embryonic pattern is characterized by three sets of melanophore stripes: along the dorsal myotomes and extending over the head, along the ventral myotomes and extending over the yolk sac, and along the horizontal myoseptum and extended ventrally under the yolk sac. Between 3 days post fertilization (dpf), when the embryonic melanophores have differentiated and migrated to their location, and 8 dpf, the embryonic pattern is very stable with almost no new addition of melanophores (Milos, Dingle and Milos 1983). After 8 dpf, as the zebrafish prepares to enter metamorphosis, a second wave of melanophores begins to populate the stripes. During metamorphosis, usually at around 14 dpf, iridophores form a line near the horizontal myoseptum, which serves as a morphological landmark to orient the developing stripe pattern. The now rapidly expanding xanthophore population is recruited to the myoseptum to develop the first adult interstripe. Concomitant with the xanthophore recruitment to myoseptum, adult melanophores begin to populate the flanks to form the first and second adult stripes. Embryonic melanophores are either repulsed by xanthophores and join the stripe, or become trapped in the expanding interstripe and die in situ. As metamorphosis progresses, the boundaries between stripes and interstripes become increasingly sharp, and the zebrafish transitions to the juvenile pattern typically around 28 dpf. The juvenile fish will have one interstripe with two
Figure 1.5. Schematic representation and images of zebrafish stripe patterning throughout development. (A) Schematic representation of the pigment patterning around the lateral myoseptum (black arrow) throughout embryonic, metamorphic and adult phases. During early metamorphosis, melanophores differentiate around the myoseptum in addition to the embryonic melanophores that persist. As xanthophores populate the developing interstripe, melanophores move to join the stripes or die (red arrows, faint grey stars). During late metamorphosis, pigment-cell density increases in the stripes and interstripes. (B) Pictures below are representative images of the embryonic, metamorphic and adult developmental stages depicted in (A) (dpf in bottom right corner).
stripes bordering it on the dorsal and ventral side of the flank. As the fish grows into adulthood, more stripes and interstripes will develop on the flanks to achieve the final adult pigmentation pattern (Figure 1.4A) (Kelsh et al. 2009; Parichy and Spiewak 2015; Singh and Nusslein-Volhard 2015).

It is worth mentioning that the characterization as detailed is a simplification of the process. Though the exact molecular details of the interaction between these cell types, and therefore the exact details of pattern formation, remain to be elucidated, zebrafish biologists have made great progress in studying the molecular requirements for interactions of these chromatophores. Further complicating pattern formation are the different subclasses of iridophores and xanthophores that can also affect the stripe patterning in a context-dependent manner. However, we are ultimately most interested in melanophore biology for the scope of this thesis. Due to their homology to human melanocytes and their relevance to disease, melanophores are the most studied of the chromatophores. Therefore, moving forward I will focus my discussions around this one pigmented cell.

1.5.3. Origins of the melanophore lineage and melanophore identity

Melanophores are derived from the neural crest. Neural crest cells, which in turn are derived from the neural tube, are highly migratory and go on to give rise to many specialized structures in the developing organism via migration, proliferation and differentiation (Mayor and Theveneau 2013). The ultimate fate of the neural crest cells is dependent on their antero-posterior position, which divides the neural crest into five categories along this axis. The neural
crest cells in the trunk group gives rise to the chromatophores and the neurons/glia (Mort, Jackson, and Patton 2015).

Cells begin to specialize within the neural crest prior to migrating away from this niche and differentiate along their migratory path to their final destination (Henion and Weston 1997), which is usually, but not necessarily, the stripe in the case of the melanophore. Notably, in zebrafish early embryonic melanophores arise from a small number of melanogenic progenitors derived directly from the neural crest without a stem cell intermediate during the first wave of melanophore population in the 2-3 dpf embryonic fish (Budi, Patterson, and Parichy 2011; Dooley et al. 2013; Hultman et al. 2009). However, melanophores that begin to populate the zebrafish flanks during metamorphosis and for the rest of its life arise from another melanophore stem cell (MSC) intermediate. It is for this reason that is possible for pigmentation mutants in zebrafish to have adult, but no embryonic pigmentation defects (Parichy and Turner 2003).

Regardless of these subtle differences in MSC, the major players in neural crest differentiation and migration are conserved across zebrafish and amniotes. Sox10 is an early marker of both melanocyte and neural lineages, and FoxD3 acts to specify a neural lineage and represses a melanocyte fate across species (Mort, Jackson, and Patton 2015; Parichy and Spiewak 2015). Furthermore, as in mammalian melanocytes, KIT also functions in the zebrafish to promote migration and survival in differentiating melanophores (melanoblast) (Rawls and Johnson 2003). In zebrafish, elegant experiments demonstrated the necessity for receptor tyrosine kinase ERB signaling for both MSC establishment and recruitment, and also allowed visualization of the MSC population in the fish (Budi, Patterson, and Parichy 2011; Hultman et al. 2009). Interestingly, mitfa-positive cells associated with the dorsal root ganglia were
identified that express neural crest marker, express melanocyte markers and were dependent on ERB signaling. The importance of mitfa is described below.

Across species microphthalmia-associated transcription factor (MITF) is a master regulator of melanocytes (Mort, Jackson, and Patton 2015). Genetic experiments in zebrafish, as well as mice, established MITF is a master transcriptional regulator of melanophores as neither fish nor mice lacking MITF can form melanocytes (Lister et al. 1999; Mort, Jackson, and Patton 2015; Steingrimsson, Copeland, and Jenkins 2004). It is worth mentioning that zebrafish have two MITF orthologues, mitfa and mitfb however as mitfb is not present in neural crest melanoblasts (Lister, Close, and Raible 2001) we will focus on mitfa. MITF transcriptional targets are associated with a range of biological processes including cellular senescence, apoptosis, proliferation, migration, and, importantly, differentiation (Yajima et al. 2011). Importantly, it has been demonstrated that MITF transcriptionally regulates three genes of a well known tyrosinase gene family involved in melanin synthesis namely, DCT, TYR and TYRP1 (Opdecamp et al. 1997; Yasumoto et al. 1997).

Melanocytes, as its name would imply, synthesize the macromolecule melanin. While birds and mammals can synthesize two kinds of melanin, eumelanin and pheomelanin, the zebrafish melanophore can only synthesize melanin (Mort, Jackson, and Patton 2015). As the biosynthetic intermediates during melanin synthesis are toxic, synthesis occurs in a specialized organelle called the melanosome. Another interesting difference between zebrafish and other species is that, while the zebrafish melanophore retains its melanosomes, in most other species the melanosome is transferred to nearby keratinocytes. However, that is not to imply that melanosomes in zebrafish are static. Melanin granules in zebrafish melanophores move along
microtubules either to disperse through the cell or to contract to camouflage the fish in a process called background adaptation (Logan, Burn, and Jackson 2006). This process can either be mediated by adrenergic cues, such as epinephrine or norepinephrine as previously discussed (Section 1.3.2), or can be mediated by hormonal pathways as well. Interestingly, the melanocortin-1 receptor (MC1R) identified as having a role in pigment dispersion downstream of melanocyte stimulating hormones (MSH), has orthologous genes in humans known to mediate pigmentation effects as well (Garcia-Borron, Sanchez-Laorden, and Jimenez-Cervantes 2005). In contrast to MSH signaling through MC1R, the Melanin-concentrating hormone (MCH), a neuropeptide expressed in central and peripheral nervous systems (An et al. 2001) was discovered to stimulate melanin aggregation in the center of the melanophore (Logan, Burn, and Jackson 2006). While this gene is conserved across species, there is no evidence for MCH having a role in mammalian pigmentation, suggesting that perhaps the function of the gene diverged.

1.5.4. Lessons learned from zebrafish pigmentation mutants

The research of vertebrate pigment patterning is a classic field of study in developmental biology. Due to their tractable genetics and striking pigmentation pattern, zebrafish are an ideal model organism in which to study pigmentation defects. Indeed, over the years molecular cloning of genes identified in zebrafish has contributed significantly to our understanding of molecular mechanisms underlying melanocyte biology. Utilizing zebrafish as a genetic tool, scientists have been able to study the role of genes in a variety of developmental pathways including differentiation, proliferation, survival, signaling and migration.
I have already mentioned a few pathways in which zebrafish genetic models were used to expand our knowledge. For example, the significance of KIT in melanogenesis has been studied in a zebrafish mutant for a KIT ortholog called sparse, in which melanophores mostly fail to migrate and undergo programmed cell death (Parichy et al. 1999). Another study found that overexpression of the Kit ligand, kitla, results in overproliferation of melanophores suggesting that further work is needed to fully understand all of the requirements for Kit signaling (Hultman et al. 2009). In addition to sparse, other zebrafish mutants such as picasso and kitzelig have been utilized to study the role of tyrosine kinases ErbB3 and ErbB2 in zebrafish orthologs erbb3b and erbb2 respectively (Hultman et al. 2009; Rojas-Munoz et al. 2009). Interestingly, mutations in erbb2 cause defects in the peripheral nervous system and lateral-line glia in addition to defects in pigmentation (Lyons et al. 2005). Findings in zebrafish mutant models of ErbB support a model in which ErbB signaling is required for the establishment of both the peripheral nervous system and the MCS in the early embryo that are later necessary to generate the adult melanophore pattern (Hultman and Johnson 2010). The nacre/mitfa zebrafish mutant, aside from increasing our knowledge in the context of the MITF gene itself, has also proven to be a tool for study in other models taking advantage of the optical clarity of these mutants for the purposes of imaging (White et al. 2008) or for screens (Iyengar, Houvras, and Ceol 2012). Recently, techniques to study melanophore-xanthophore signaling and motility in vitro were developed (Yamanaka and Kondo 2014), further improving the repertoire of experiments possible in zebrafish models.

Taken together, these experiments demonstrate that the study of pigmentation patterning can yield key contributions to biology. Zebrafish pigmentation mutants offer a tractable model in which to elucidate molecular mechanisms behind melanocyte behavior.
1.6. References


Yamanaka, Hiroaki, and Shigeru Kondo. 2014. 'In vitro analysis suggests that difference in cell movement during direct interaction can generate various pigment patterns in vivo', *PNAS*, 111: 1867-72.


Chapter Two

Transgenic expression of \( GNAQ/11^Q209L \) in cooperation with \( tp53 \) mutation drives a novel zebrafish model of Uveal Melanoma

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Work to generate the zebrafish model of UM was done by Adam Amsterdam and Andrea Henle. Kaplan-Meier Curves were generated by Adam Amsterdam. Characterization of pigmentation defect and IHC was done by Dahlia Perez. RNA from tumor samples were generated by Dahlia Perez and Adam Amsterdam. Charlie Whittaker performed analysis of RNAseq data sets.
Chapter 2: Table of Contents

2.1. Abstract .............................................................................................................54

2.2. Introduction ........................................................................................................55

2.3. Results ...............................................................................................................58

2.3.1. Generation of zebrafish model of uveal melanoma using Tol2-mediated
       transgenesis to express human oncogenic GNAQ behind a mitfa-
       promoter ...........................................................................................................58

2.3.2. GNAQ/11Q209L oncogenic mutation cooperates with tp53 mutation in
       transgenic zebrafish to develop aggressive pigmented tumors .................61

2.3.3. Melanomas in Tg(mitfa:GNAQ/11Q209L); tp53M214K/M214K zebrafish
       recapitulate human uveal melanoma YAP signaling ..................................65

2.4. Discussion .........................................................................................................67

2.5. Methods ............................................................................................................70

2.6. Supplemental Figures and Tables ..................................................................75

2.7. References ........................................................................................................77
2.1. Abstract

Uveal melanoma (UM) is the most common primary intraocular cancer in humans. Though primary tumors are treatable, approximately 50% of patients diagnosed with UM develop liver metastases within 15 years, after which median survival is only 6 months. As there are no treatment options for the metastatic disease, further work to discover new therapeutics and understand disease initiation and progression is crucial. 83% of UM tumors contain activating mutations in one of two genes encoding Gα proteins, GNAQ or GNA11, associated with G-protein coupled receptors. In the vast majority of cases, the GNAQ/11 mutations alter codon Q209, which lies within the GTPase domain, resulting in constitutive activation. We have developed a zebrafish model of UM using Tol2-mediated transgenesis to insert human GNAQ/11Q209L tumor alleles into the zebrafish genome under the control of melanocyte-specific promoter mitfa. These Tg(mitfa:GNAQ/11Q209L) fish develop both uveal and cutaneous melanomas with nearly complete penetrance upon addition of cooperating mutation tp53M214K shortening the lifespan of these animals. These tumors recapitulate the human disease in that they express high levels of the human transgene and increased expression of YAP. Furthermore, these mutants display a range of internal and external pigmentation defects including hyperpigmentation and mislocalization of melanophores.
2.2. Introduction

Uveal melanoma (UM), an intraocular malignancy arising in non-classical melanocytes found in the choroid, iris and ciliary body, is a highly aggressive cancer with no effective treatment strategies for the metastatic disease (Shoushtari and Carvajal 2014). Within 15 years of primary diagnosis approximately 50% of UM patients will present with metastases, at which point median survival drops to just 6 months following diagnosis. In the past decade, large-scale UM sequencing projects have uncovered recurrent, mutually exclusive mutations in two homologous proteins, GNAQ and GNA11, in over 80% of UMs (Onken et al. 2008; Van Raamsdonk et al. 2009; Van Raamsdonk et al. 2010). GNAQ and GNA11 are 90% homologous at the amino acid level and will consequently be referred to as GNAQ/11. GNAQ/11 encode the α-subunits of a heterotrimeric G-protein receptor (GPCR) complex, and they function to transduce signals from a GPCR to downstream effectors within the cell (Rozengurt 2007). GNAQ/11 is known to signal directly to phospholipase C (PLC), which can lead to activation of MAPK cascade or NFKB signaling through activation of protein kinase C (PKC) (Lee et al. 1992; Van Raamsdonk et al. 2009; Zuidervaart et al. 2005; Wu et al. 2012).

Hotspots for oncogenic mutation are at the Q209 and R183 residues located in the Ras-like GTPase domain of GNAQ/11 (Van Raamsdonk et al. 2009; Van Raamsdonk et al. 2010). In the wild-type context, GNAQ/11 remain GDP-bound in an inactive state, unless stimulated by a ligand-activated GPCR to release GDP and instead bind GTP, thereby assuming an active conformation to interact with downstream effectors of the signaling pathway. Typically, the Ras-like GTPase domain assists in hydrolyzing the GTP back to GDP to return GNAQ/11 to the inactive state (Markby, Onrust, and Bourne 1993). However, the oncogenic mutations in the Ras-
like GTPase drastically reduce the rate of GTP hydrolysis thereby maintaining GNAQ/11 in a constitutively activated state (Landis et al. 1989). Interestingly, oncogenic GNAQ/11 can activate YAP signaling by facilitating YAP nuclear translocation via a HIPPO-independent pathway indicating there may be distinct pathways activated by oncogenic GNAQ/11 not activated by wild-type proteins (Feng et al. 2014; Yu et al. 2014).

Though our understanding of the genetic drivers of UM has progressed enormously in the past decade, many challenges still preclude the translation of these findings into clinically relevant results (Shoushtari and Carvajal 2014). One major limiting factor is the scarcity of cell lines and patient samples, due to the rarity of the disease. Therefore, the development of animal models of UM is necessary for the continuing study of this malignancy. To this end, we present a zebrafish model of UM driven by melanocyte-specific expression of oncogenic GNAQ/11Q209L.

The zebrafish is now a well validated model organism for cancer research. Zebrafish offer a variety of technical benefits over other vertebrate models including: tractable genetics, high fecundity, relatively small maintenance costs, and amenability to various types of screens (Mione and Trede 2010; White, Rose, and Zon 2013). Furthermore, several oncogenic mutations in human cancers are now known to have similar tumor phenotypes in zebrafish (Amsterdam et al. 2004; Langenau et al. 2003; Patton et al. 2005). Importantly for a melanoma model, we recognize the high level of homology between zebrafish melanophores and human melanocytes, and that a cutaneous melanoma model driven by oncogenic BRAFV600E expression in cooperation with tp53 mutation has been successfully modeled in zebrafish (Patton et al. 2005). Though mutations in the well known tumor suppressor gene tp53 are surprisingly rare in both cutaneous and uveal melanomas (Polsky and Cordon-Cardo 2003), tumors driven by tp53 loss of function
display a high degree of aneuploidy (Zhang et al. 2013) and therefore *tp53* loss of function represents a reasonable cooperating mutation to facilitate tumorigenesis. To cooperate with oncogenic *GNAQ/11Q^{209L}* in our zebrafish model of UM, we specifically introduced the frequently used *tp53^{M214K/M214K}* mutation discovered by Berghmans and colleagues that gives rise to malignant nerve sheath tumors (MPNST) (Berghmans et al. 2005).

Taken together, we believe that a zebrafish model of UM will complement existing models and further expand our understanding of the disease.
2.3. Results

2.3.1. Generation of a zebrafish model of uveal melanoma using Tol2-mediated transgenesis to express human oncogenic GNAQ behind a melanocyte-specific promoter

Since oncogenic GNAQ/11 mutations account for over 80% of initiating mutations in UM, we used Tol2-directed transgenesis to insert the cDNA of either human \( GNAQ^{Q209L} \) or \( GNA11^{Q209L} \) tumor alleles into the zebrafish genome under the control of the melanocyte-specific promoter, \( mitfa \) (Figure 2.1A). Embryos were injected at the single-cell stage, and raised until adulthood. The resulting chimeric zebrafish had significant hyperpigmentation defects with large patches of hyperpigmentation, presumably in the cells expressing the oncogene (Figure 2.1B).

We screened the chimeras for germline transmission to recover stable zebrafish lines expressing the \( GNAQ^{Q209L} \) or \( GNA11^{Q209L} \) transgene in melanocytes to study the effect of the oncogenic \( GNAQ/11 \) alleles in vivo. Single copy integration of the transgene was verified by Southern blot (data not shown). In total, 11 \( GNAQ^{Q209L} \) lines and 9 \( GNA11^{Q209L} \) single integrant lines were recovered.

In all of the stable \( GNAQ/11^{Q209L} \) transgenic lines, the juvenile and adult transgenic zebrafish (\( Tg^+ \)) displayed clear pigmentation defects compared to their clutchmate controls (Figure 2.2), which typically worsened with age (data not shown). First, we observed hyperpigmentation and nonlinear stripes in the skin (Figure 2.2A). Second, in a partially penetrant manner, the choroid of \( Tg^+ \) animals, but never the retinal pigmented epithelium (RPE), was thickened (Figure 2.2B). Finally, the \( Tg^+ \) zebrafish displayed internal pigmentation phenotypes in a range of organs and tissue types. In particular, the \( Tg^+ \) animals frequently had rings of hyperpigmentation around blood vessels (Figure 2.2C) or organs (data not shown).
Additionally, pigment was present in the musculature where it is rarely, if ever, seen in wild-type clutchmates (Figure 2.2D).

Figure 2.1. Generation of zebrafish with melanocyte specific expression of GNAQ/11Q^{209L}. (A) Schematic of transgene insert. (B) Chimeric zebrafish generated by Tol2-mediated GNAQ^{Q209L} transgene insertion during embryogenesis display a hyperpigmentation phenotype (b''',b'''') compared to wild type animals (b').
Figure 2.2 Zebrafish with melanocyte specific expression of GNAQ/11^{Q209L} have pigmentation defects. Compared to clutchmate controls, juvenile/adult *Tg(mita:GNAQ^{Q209L})* zebrafish (*Tg^{+}*) display (A) hyperpigmented skin with disorganized stripes, (B) thickening of the choroid layer (blue arrow), but not the RPE (green arrow), (C) hyperpigmentation around the blood vessels of the gills, and (D) pigmentation in the musculature. Scale bar=1cm.
2.3.2. \textit{GNAQ/11Q^{209L}} oncogenic mutation cooperates with \textit{tp53} mutation in transgenic zebrafish to develop aggressive pigmented tumors

We aged the \textit{GNAQ/11Q^{209L}} zebrafish lines and found that the \textit{Tg}^{+} fish developed tumors at low incidence and with long latency (greater than 18 months; data not shown). This was unsurprising, as tumor development typically requires more than a single oncogenic event. Therefore, to promote tumor formation, we crossed three of our \textit{Tg(mitfa:GNAQ^{209L})} lines and four \textit{Tg(mitfa:GNAJ^{209L})} lines with fish carrying a \textit{tp53} mutant allele, \textit{tp53}^{M214K} (Berghmans et al. 2005), which are highly predisposed to develop malignant nerve sheath tumors (MPNST). We then generated cohorts of fish that were \textit{Tg}^{-} or \textit{Tg}^{+} and either heterozygous or homozygous for \textit{tp53}^{M214K}. These fish were aged to assess time to morbidity. In both the \textit{tp53}^{M214K/+} and \textit{tp53}^{M214K/M214K} backgrounds, we found that the \textit{Tg}^{+} fish died significantly early than their \textit{Tg}^{-} clutchmate controls (Figure 2.3A,B). The decreased survival varied from line to line, but was significant for all seven \textit{Tg(mitfa:GNAQ/11Q^{209L})} lines (Figure 2.3B). Consistent with the fact that \textit{GNAQ^{209L}} versus \textit{GNAJ^{209L}} appear synonymous in the initiation of human UM, the \textit{GNAQ^{209L}} and \textit{GNAJ^{209L}} lines showed comparable phenotypes with regard to the kinetics of mortality (compare red versus blue curves in Figure 2.3B).

Most importantly, this accelerated mortality was accompanied by a switch in tumor spectrum; the \textit{Tg}^{-} controls developed MPNSTs characteristic of the \textit{tp53}^{M214K} mutant strain whereas the \textit{Tg}^{+} clutchmates developed melanotic tumors with complete, or near complete, penetrance. The tumors were highly heterogenous, typically including both pigmented regions and unpigmented areas suggestive of a poorly differentiated state. They were also extremely aggressive, with regard to both speed of growth and ability to invade into surrounding tissues.
Figure 2.3. Oncogenic GNAQ cooperates with \( tp53 \) mutation to give rise to pigmented tumors. (A) Kaplan-Meier curves for one \( Tg(\text{mitfa}:GNAQ^{209L}) \) line (Q-1) comparing the survival of \( Tg^{+};tp53^{M214K/M214K} \) fish (red line labeled \( Q-1;tp53^{+/-} \)) compared to clutchmate \( Tg^{+};tp53^{M214K/M214K} \) controls (black line labeled \( tp53^{-/-} \)), and also \( Tg^{+};tp53^{M214K/+} \) fish (blue line labeled \( Q-1;tp53^{+/-} \)) compared to \( Tg^{+};tp53^{M214K/+} \) (grey line labeled \( tp53^{+/-} \)) controls. (B) Comparison of the survival of fish that are \( tp53^{M214K/M214K} \) and carry distinct \( \text{mitfa}:GNAQ^{209L} \) (red lines labeled \( Q^{+};tp53^{+/-} \)) or \( \text{mitfa}:GNAQ^{117L}^{209L} \) (blue lines labeled \( 11^{+};tp53^{+/-} \)) transgenic insertions versus their combined \( tp53^{M214K/M214K} \) controls (black line labeled \( tp53^{-/-} \)). Representative H&E stained sections of (C) a choroidal melanoma (right) versus a wild-type eye (left) with a healthy choroid (blue arrow) and RPE (green arrow); (D) an internal melanoma located within the abdomen; and (E) a cutaneous melanoma (100\( \mu \)m scale). For brevity, \( tp53^{M214K/M214K} \) is labeled as \( tp53^{+/-} \).
Tumors arose in a variety of anatomical locations. The most prevalent was cutaneous melanoma (Figure 2.3E), which is consistent with the existence of GNAQ/1Q209L mutations within a small fraction of human cutaneous melanomas (Johnson et al. 2016). Importantly, we also observe ocular tumors (Figure 2.3C and Supplemental Figure S2.1). Analyses of H&E-stained sections of smaller, emerging eye tumors show that these arise from the choroid layer and their histology closely resembles human uveal melanomas (personal communication, Dr. Stacey, Massachusetts Eye and Ear Infirmary). Finally, we often observed melanotic tumors within internal tissues including the musculature, liver, pancreas and heart (Figure 2.3D, Supplemental Figure S2.2). These internal tumors are consistent with the presence of abnormal internal pigmentation within the Tg+ zebrafish (Figure 2.2). As anticipated, genotyping of tumors arising in Tg+:tp53M214K/+ zebrafish showed that these had undergone loss of the wild-type allele of tp53 (data not shown).

To assess expression of the GNAQ transgenes, we performed immunohistochemistry on sections taken from ocular and internal tumors arising in Tg(mitfa:GNAQQ209L);tp53M214K/M214K fish from the Q-1 line using an antibody that recognizes an epitope in human GNAQ that is poorly conserved in zebrafish GNAQ (Figure 2.4A,B). This yielded a strong signal in the uveal melanoma and heterogenous staining of the pigmented liver tumor, with little or no staining of control wild-type eye or liver (Figure 2.4A,B). We also took advantage of transcriptomic sequencing data to compare the total levels (ie human and zebrafish) of GNAQQ209L mRNA present in uveal and cutaneous melanomas taken from Tg(mitfa:GNAQQ209L);tp53M214K/M214K zebrafish (n=5) versus cutaneous melanomas from Tg(mitfa:BRAFV600E);tp53M214K/M214K fish.
Figure 2.4. GNAQ is overexpressed in pigmented tumors in Tg(mitfa:GNAQ<sup>Q209L</sup>);tp53<sup>M214K/M214K</sup> zebrafish. H&E and immunohistochemical staining of human GNAQ in sequential sections from (A) ocular tumors and (B) liver tumors. Black dotted lines delineate liver tissue in wild-type control. Scale bar = 100 μm. For brevity, tp53<sup>M214K/M214K</sup> is labeled as tp53<sup>-/-</sup>. (C) Reads aligning to human oncogenic GNAQ, with or without mismatches to also allow detection of the endogenous zebrafish GNAQ, show greatly increased levels of GNAQ mRNA in Tg(mitfa:GNAQ<sup>Q209L</sup>) driven melanomas versus BRAF<sup>Y600E</sup> driven melanomas in cooperation with tp53<sup>-/-</sup> mutation.
(n=3). Reads in the $Tg(mitfa:GNAQ^{Q209L})$ driven melanomas aligned perfectly to the human GNAQ$^{Q209L}$ sequence (Supplementary Figure S2.3) and were present at approximately 100-fold higher levels than the zebrafish GNAQ-aligning reads present in the $BRAF^{F^600E}$ driven melanomas (Figure 2.4C). Collectively, these data verify elevated GNAQ$^{Q209L}$ within the tumors of the $Tg(mitfa:GNAQ^{Q209L})$ Q-1 transgenic line.

2.3.3. Melanomas in $Tg(mitfa:GNAQ/11Q^{209L});tp53^{M214K/M214K}$ zebrafish recapitulate human uveal melanoma YAP signaling

YAP translocation to the nucleus downstream of oncogenic GNAQ signaling via a HIPPO-independent pathway has been previously described (Feng et al. 2014; Yu et al. 2014). In human UM samples, nuclear localization of YAP has been shown to correlate with GNAQ/11 mutation (Yu et al. 2014). To determine whether our zebrafish UM model recapitulates this hallmark of human UM, we performed immunohistochemistry on tumors derived from $Tg(mitfa:GNAQ^{Q209L});tp53^{M214K/M214K}$ fish from the Q-1 line using an anti-human YAP antibody whose epitope is partially conserved in zebrafish YAP (Figure 2.5). This antibody yielded a strong signal that was contained within the tumors and not the surrounding healthy tissue (Figure 2.5). Moreover, high magnification shows that this is localized to the nucleus (Figure 2.5, right panel). These data confirm that YAP is activated within our oncogenic GNAQ-driven melanomas, phenocopying the signaling events that are activated within human UM. Therefore, we believe that analysis of this zebrafish model can yield insight into the central mechanism(s) underlying the development of UM.
Figure 2.5. YAP is overexpressed and localized to the nucleus in Tg(mita:GNAQ^{Q209L});tp53^{M214K/M214K} pigmented tumors. Immunohistological staining of tumors from Tg(mita:GNAQ^{Q209L});tp53^{M214K/M214K} zebrafish confirms overexpression (center panel) and nuclear localization (right panel) of YAP compared to wild type controls (left panel). Scale bars = 200μm, 500μm, 100μm from top to bottom panels respectively. For brevity, tp53^{M214K/M214K} is labeled as tp53^-/-.
2.4. Discussion

Studies in the past decade have elucidated the genetic drivers of UM; however, more research and tools are still required to advance our knowledge of this disease in a clinically relevant way. To this end, we have successfully generated a novel zebrafish model of UM driven by transgenic expression of the initiating mutations GNAQ/11 in cooperation with *tp53* mutation. Previous efforts to study UM have been limited to cell culture-based work or orthotopic injection of UM cell lines. Our transgenic model circumvents the disadvantages inherent in working only in an *in vitro* context or in an *in vivo* context with a suppressed immune system as in interspecies xenograft models. Furthermore, the observation that these tumors overexpress and translocate YAP to the nucleus lends confidence that our model is accurately recapitulating human UM also at a molecular level and could be a valuable genetic tool for future studies.

As predicted, we find that the transgenic fish develop ocular tumors, but interestingly the melanomas formed are not limited to the eye. We demonstrate that the *Tg(mitfa:GNAQ/11Q209L);tp53*M214K/M214K* fish develop a range of aggressive, melanized tumors in tissues including but not limited to the: liver, pancreas, kidney, skeletal muscle and skin; and that the consequence of these tumors is a shortened lifespan in zebrafish carrying the transgene and *tp53* mutation. The formation of cutaneous melanomas is not wholly unexpected as mutations in GNAQ/11 are found, albeit infrequently, in cutaneous melanomas in human patients (~5%)(Shoushtari and Carvajal 2014). However, the presence of these internal and cutaneous, or otherwise non-ocular, tumors raises many questions. Often a transgenic zebrafish will have multiple tumors at the time of death; as such an important question to answer is: do these different tumors represent distinct melanoma initiating events or are they metastases from the same tumor? Around 90% of
metastatic human UMAs metastasize to the liver, in this model we do see melanomas in the liver, sometimes as the only tumor in the fish and sometimes as one of many. However, even if there are multiple tumors in the fish, we do not yet know whether these tumors are the result of metastases. Indeed, typically when the zebrafish must be sacrificed the tumor has invaded so many organs that identifying the origin of the tumor is not possible. This question is also complicated by the presence of extraneous internal pigmentation in the mutant fish. This phenotype renders it formally possible that these pigmented areas represent a cell competent to initiate a tumor. Indeed, this may be the more likely scenario as we observe internal tumors that have no contact with the skin, therefore they have likely arisen from internal melanocytes or melanocyte precursors.

To expand our understanding of these tumors in multiple tissues, we are currently generating RNAseq data for a large sampling of UM tumors from our model using a platform for massive parallel sequencing called 3'-tag digital gene expression (DGE) (Asmann et al. 2009). We have completed some initial sequencing, and determined that we would achieve greater significance in our analysis by sequencing more samples, which is often the case in heterogeneous tumor populations. Through sequencing more tumors, we will hopefully be able to draw comparisons between tumors in different tissues. Understanding the transcriptome differences between a tumor in the eye compared to a tumor in the liver or skin, for example, could help us understand the relevance of the tumor microenvironment in UM tumors. In addition to interrogating transcriptional changes in tumors in different locations, we also plan to compare the transcriptional programs in our cutaneous tumors driven by oncogenic GNAQ/11 expression to BRAF-driven cutaneous tumors previously reported (Patton et al. 2005). Through
this analysis we hope to draw a direct comparison between the effect of these two distinct oncogenic drivers in melanomas.

Furthermore, upon completion of RNAseq analysis we could identify genes that share expression patterns in our tumors and in publicly available human UM datasets. As previously discussed, the zebrafish is an excellent model organism in which to quickly study and screen candidate cooperating mutations, a feature that represents one of the main advantages to working in murine models of UM and other diseases. Therefore, our ultimate goal is to use our zebrafish model of UM; first, to discover additional genes involved in UM tumorigenesis, and second to perform genetic screens as functional assays on these genes to further our knowledge of the disease.
2.5. Methods

Zebrasfish lines and UM model generation

Experiments were performed in the AB/Tübingen zebrafish genetic background known as TAB5/14 generated by Nancy Hopkins and Adam Amsterdam (Amsterdam et al. 1999).

For the generation of the transgenic lines, GNAQ/11Q209L human cDNA clones were purchased from MS&T either with or without an epitope (EE) tag (Peavy et al. 2005). GNAQQ209L clones purchased were as follows: GNAOQ000C0 and GNAOQ0EIC0 (with EE tag). GNA11Q209L clones purchased were as follows: GNA11000C0 and GNA110EIC0 (with EE tag). To make the Tol2 constructs for transgene insertion into the zebrafish genome, the mitfa promoter (Patton, 2005) and the GNAQ/11 full length ORF were directionally cloned into the Gateway Tol2 vector pTolDestR4-R2pA. The Tol2 vector and mRNA encoding the Tol2 transposase were microinjected into one-cell TAB5/14 zebrafish embryos. For generation of the UM model, Tg(mitfa:GNAQ/11Q209L) founders were crossed directly into the tp53M214K/M214K mutant background. F0 animals were raised and mated, the resulting F1 embryos were identified by PCR of DNA isolated from fin biopsies after at least 6 weeks of age, and subsequently analyzed for germline transmission. Up to 100 progeny were screened per potential founder by extracting genomic DNA from pools of 5-7 3 dpf embryos for analysis by PCR for the GNAQ/11 transgene. Once founders were identified, southern blots were performed on the tails of transgenic F1 fish to confirm single-copy integration, in total 11 GNAQQ209L and 9 GNA11Q209L single-copy mutant lines were recovered.

For genotyping, genomic DNA was extracted from adult fish tail clip obtained from fish anesthetized using 0.05% Tricaine or euthanized embryos. Tail clippings or embryonic fish were
incubated at 56°C overnight in 100mM Tris pH 8.3, 200mM NaCl, 5mM EDTA, 0.4%SDS solution for DNA extraction. The genotyping for the GNAQQ209L mutation used transgene-specific PCR with the following primers: forward (5’- CAG AAC ATC TTC ACG GCC ATG – 3’) and reverse (5’- TCA GCT ACG CGG TCC AAG TC -3’). The genotyping for the GNAI1Q209L mutation used transgene-specific PCR with the following primers: forward (5’- GCC AAT GCG CTC CTG ATC CG – 3’) and reverse (5’- CAA GGT GGC GAT GCG GTC AAC – 3’). Primers against Wnt were used as a positive control in the same reaction: forward (5’- CAG TTC TCA CGT CTG CTA CTG GCA -3’) and reverse (5’-ACT TCC GGC GTG TTG GAG AAT TC -3’). Genotyping for tp53M214K mutation used allele-specific PCR with a single forward primer and two reverse primers to detect the mutant and the wild-type alleles as previously described (Berghman, 2005).

Imaging zebrafish

For live imaging, fish were anesthetized in 0.05% tricaine and immobilized in 3% methylcellulose prior to imaging on a backlit stereomicroscope. Images were taken with a SPOT insight color camera model 3.2.0 and processed with SPOT image analysis software. Images of H&E or IHC stained slides were taken on a Nikon Eclipse Ds-Ri2 microscope and camera and processed with NIS-elements software V4.3.

Tumor onset analysis

Tumor onset analysis was performed in 3 Tg(mita:GNAQQ209L) and 4 Tg(mita:GNAI1Q209L) lines in either tp53M214K/M214K or tp53M214K/+ mutant backgrounds.
Clutchmate controls did not carry the transgenes, but were tp53<sup>M214K/M214K</sup> or tp53<sup>M214K/+</sup> mutants. Genotypes were determined by PCR as described above, and fish were maintained in tanks at similar densities to minimize environmental effects. Zebrafish were euthanized upon signs of ill health, including life-threatening tumors, in compliance with CAC approved protocols. Kaplan-Meier curves were created in Prism.

**Immunohistochemical staining**

Fish were euthanized in 0.1% tricaine and then placed in 4% paraformaldehyde at 4°C overnight for fixation. To de-calcify the fish they were transferred to 0.50M EDTA (pH 8.0) for 7 days. The fish were dehydrated in alcohol and submitted to the KI Swanson Biotechnology Core Facilities for paraffin embedding. Tissue sections (4 μm thick) from paraffin-embedded tissue blocks were placed on charged slides, deparaffinized in xylene, rehydrated through graded alcohol solutions, and either stained with hematoxylin/eosin or were left unstained for immunohistochemical staining.

For immunohistochemistry, after deparaffinization and rehydration, slides were boiled in antigen retrieval solution (1.8mM citric acid, 8.2mM sodium citrate). High levels of melanization in tumors required that slides be bleached prior to antibody staining, melanin bleaching was performed in 3% hydrogen peroxide in PB buffer (45mM NaH₂PO₄H₂O, 154mM Na₂HPO₄). Staining conditions were as follows: blocking in 5% goat serum; primary antibody incubation overnight at 4°C using YAP antibody #4912 (Cell Signaling) at 1:200 dilution or GNAQ antibody ab75825 (Abcam) at 1:700 dilution; secondary anti-Rb antibody at 1:200 dilution followed by DAB reaction.
Nextseq sequencing and data processing

For RNA extraction, moribund Tg(mita:GNAQ^{Q209L});tp53^{M214K/M214K} or Tg(mita:BRAF^{V600E});tp53^{M214K/M214K} fish (Patton et al. 2005) were euthanized and tumors quickly dissected. In total, RNA was isolated from 8 melanomas: 5 eye or cutaneous Tg(mita:GNAQ^{Q209L});tp53^{M214K/M214K} tumors and 3 cutaneous Tg(mita:BRAF^{V600E});tp53^{M214K/M214K}. RNA from the dissected tumor tissue was isolated either with Trizol reagent (Invitrogen) or Qiagen RNeasy Minikit.

RNAseq libraries were prepared with Clontech Smartseq V3 library kit using the following conditions: 14 cycles PCR cDNA synthesis, fragmentation by sonication and Spriworks robotic library production for end repair, a-tailing, ligation, size selection. Samples were quantified on advanced analytical fragment analyzer and by QPCR prior to sequencing. Samples were sequenced on NextSeq500, V2 chemistry with 75-75 paired-end sequencing.

To test for the presence of Tg(mita:GNAQ^{Q209L}) transgene RNA in the tumors, all reads from the tumor samples were aligned to the human GNAQ sequence (see Supplemental Figure 2.3) using bwa mem aligner. Reads were extracted from the full output and stratified according to number of mismatches. Only Tg(mita:GNAQ^{Q209L});tp53^{M214K/M214K} samples had no mismatched reads, alignments with mismatches in the tumors from the Tg(mita:BRAF^{V600E});tp53^{M214K/M214K} aligned again to the Zf10 genome assembly to confirm that they were endogenous transcripts.
2.5.1. Acknowledgements

We thank all members of the Lees lab for their constant input throughout this study. This work was partially funded by the Melanoma Research Alliance. Dahlia Perez was supported by an NSF fellowship and by D.H. Koch internal fellowship award.
2.6. Supplementary Figures and Tables

Supplemental Figure S2.1. *Tg(mif4a:GNAQQ209L);tp53M214K/M214K* zebrafish develop ocular tumors. Images show 3 zebrafish with varying ocular tumors.

Supplemental Figure S2.2. *Tg(mif4a:GNAQQ209L);tp53M214K/M214K* zebrafish develop a wide variety of tumors. (A) H&E staining of various melanized tumors in the *Tg*+;*tp53M214K/M214K* abdominal cavity. Tumor can be found invading many tissues including the liver, pancreas, kidneys and tissue surrounding the heart. Degree of pigmentation in the tumors varies widely as well. (B) H&E staining of tumor tissue invading skeletal muscle of a *Tg*+;*tp53M214K/M214K* zebrafish.
>hs_gnaq.fa
ATGACTCTGGAGTCATCATGGCGTGCTGCTGAGCGAGGCCAAGGAGCCCGGCAGATCAACGACGAGATC
GAGCG
GCAGCTCCGAGGGACAAGCGGGACCGCCCGCCGGAGGCTCAAGCTGCTGCTGCTCCAGACGGAGAGAGATG
AGAGTA
CGTTTATCAAGCAGTGAATCATCCATGGGTCAGGATACTCTGATGAAAGATAAAGGGCTTCAACCAAGCTGAG
TAT
CAGAACAATCTTCTCACGGCCATGCGACACTGACATTTTCAAGATCTAATGAGAGAAGCTTGAGAGAG
TAA
GGCTCAGCAAAATTAGGTCCGAGAAGTGGTGTGGAAGGTTGCTCTTTTGGAAATCCATAGTATGAGCAAT
AGA
GTATTGGAAATGATCCTGGATCCAGAAATGCTATGAGACGACAGAATCAAAATTATCGGCTCAACAA
ATT
CTTAATGACTTTGGACCCGCTAGCTGACCCCTTGATACATGCTACGGAACACAGATGCTCTAGTCAGTTCC
CAC
GGGATCATGGATGCAATACCCCTTTGACTTACAAAGTGTCTTTTCAGAATGGTCGATGAGGGGGCTAAAGG
TCAGAGA
GAA
GAAATTGGATACACTGCTTTGGAAATGTCACCTCTACATGTTCTTCTAGTCAGCTTATGGAATATGAGGAC
TCTGG
GAGTCAGACAATGAGAACCAGATGAGAGACGGCTCTCTTTTACAAATTCACATACCCTGGITCCAGA
ACTC
CTCGTTATCTGTCTTTAAACAAAGAGATCTCTAGGGAAGAAATCATGTATCTCCACTGACTCTCC
AG
AAATATGAGGACCCGAGATGCCACGGGGACAGATTCATTGTGAAGATGTGGACCGAGAACCAGA
CAGT
GACAAAAATTATCTACCTCCACTTACGCTGAGCAGCACCAGAATATCCTCCCTTTGTCTTTGCTGCC
ACAC
CATCTCCAGTGGAAACCTGAAGGAGTACAATCTGCTTGCTAA

Supplemental Figure S2.3. Human GNAQ sequence used for alignment in determining transgene transcript levels in Tg(mifa:GNAQ<sup>Q209L</sup>);tp53<sup>M214K/M214K</sup> tumors.
2.7. References


Chapter Three

Expression of oncogenic GNAQ/11 disrupts melanocyte biology in a zebrafish model of uveal melanoma

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All *in vivo* and *in vitro* work was conducted by Dahlia Perez. The transgenic model had been previously generated by Adam Amsterdam and Andrea Henle. Pictures of fins in GNAQ transgenics were taken by Andrea Henle. Both Adam Amsterdam and Hannah Hagen assisted in the metamorphic patterning experiment. Charlie Whittaker processed the RNAseq data.
Chapter 3: Table of Contents

3.1. Abstract.................................................................................................................82

3.2. Introduction............................................................................................................83

3.3. Results.......................................................................................................................87

3.3.1. Tg(mitfa:GNAQ/11Q209L) transgenic zebrafish display in vivo pigmentation defects throughout development.........................................................87

3.3.2. Background adaptation assays allow for quantitation of pigmentation defects in Tg(mitfa:GNAQ209L) zebrafish.................................................................89

3.3.3. Tg(mitfa:GNAQ209L) expression increases melanin distribution without increasing melanophore size.................................................................91

3.3.4. Tg(mitfa:GNAQ209L) expression in melanophores results in increased dendricity and altered morphology.................................................................94

3.3.5. Tg(mitfa:GNAQ209L) expressing melanophores have increased survival capacity.................................................................................................................98

3.3.6. Analysis of differential expression patterns of transgenic versus non-transgenic melanophore populations.................................................................100

3.4. Discussion................................................................................................................110

3.5. Methods...................................................................................................................115

3.6. Supplemental Figures and Tables...........................................................................122

3.7. References...............................................................................................................132
3.1. Abstract

In Chapter 2, we present a novel zebrafish model of uveal melanoma (UM) driven by human GNAQ/11Q209L tumor alleles under the control of melanophore-specific promoter mitfa in cooperation with tp53M214K/M214K mutation. We report internal and external pigmentation defects in Tg(mitfa:GNAQ/11Q209L) zebrafish compared to clutchmate controls. Here we utilize our Tg(mitfa:GNAQ/11Q209L) zebrafish, independent of tp53 mutation, to study how oncogenic expression of GNAQ/11 disrupts melanophore biology. Characterization of the transgenic fish reveals that phenotypes including hyperpigmentation, mislocalization of melanophores and failure to respond to boundary cues are quantifiable as early as 3 days post fertilization and persist throughout the development of the fish. We utilize a background adaptation assay to facilitate quantitation of Tg(mitfa:GNAQ/11Q209L) phenotypes and demonstrate that hyperpigmentation is due in part to an increase in melanophore number and an increase in melanin distribution per melanophore. To expand beyond morphological analysis, we develop methods of melanophore isolation for in vitro culture and RNAseq. We observe that transgenic melanophores in vitro are more dendritic, motile and better survivors that non-transgenic controls. Whole transcriptome analysis of these melanophore populations yields a list of 353 differentially expressed genes. Functional analysis of this gene set reveals changes in categories related to axon guiding, cell adhesion and cell cycle regulation. Taken together, these experiments implicate oncogenic GNAQ/11 in cell proliferation, cell-signaling, cell motility and cell survival. The goal of this research is to ultimately understand the mechanisms by which oncogenic GNAQ mediates these phenotypes in the context of UM.
3.2. Introduction

As previously introduced, uveal melanoma (UM) is a unique type of melanoma arising from nonclassical melanocytes in the eye, with mutations in homologous proteins GNAQ or GNA11 in over 80% of tumors (Van Raamsdonk et al. 2010; Onken et al. 2008). GNAQ/11Q209L represents the most common mutation, and results in constitutive activation of GNAQ/11, which encode the α-subunits of a heterotrimeric G-protein receptor (GPCR) complex.

GNAQ/11Q209L is known to signal to a diverse set of downstream pathways, the best characterized of which is phospholipase C beta-1 (PLC) (Lee et al. 1992). PLC itself has a diverse set of downstream effectors, such as activation of the MAPK cascade resulting in high phosphorylated MEK and ERK levels (Van Raamsdonk et al. 2009; Zuidervaart et al. 2005). Oncogenic GNAQ/11Q209L specifically activates YAP signaling via a HIPPO-independent pathway (Feng et al. 2014; Yu et al. 2014). A potential mechanism for this activation involves actin polymerization downstream of GNAQ/11Q209L signaling through Trio/Rho/Rac (Yu et al. 2014), suggesting that oncogenic GNAQ/11 expression activates changes in the actin cytoskeletal network.

While evidence of the signaling pathway dysregulated downstream of oncogenic GNAQ/11 in the context of UM has been studied in transformed melanocytes, cultured human UM tumor cell lines or xenograft and tumor models (Van Raamsdonk et al. 2009; Mueller et al. 2002; Yu et al. 2014; Shoushtari and Carvajal 2014), relatively little is known about how oncogenic GNAQ/11 signaling affects the fundamental biology of the melanocyte. To this end, we utilize our transgenic zebrafish model of UM, driven by melanocyte specific expression of GNAQ/11 oncogenic alleles, to assess the behavior of untransformed melanocytes upon expression of
oncogenic $GNAQ/11^{Q209L}$. The zebrafish homolog of the human melanocyte, called the melanophore, is well characterized and therefore represents a classic model in which to interrogate aberrant behavior as a result of oncogenic GNAQ/11 expression. Many of the developmental programs involved in melanophore differentiation are conserved between zebrafish and amniotes (Mort, Jackson, and Patton 2015). Indeed, research on zebrafish stripe patterning has generated significant contributions to our understanding of mechanisms underlying melanocyte biology.

The striped pigment pattern of zebrafish comprises three types of pigment cells, melanophores (the melanocyte homolog) and xanthophores (yellow chromatophore), and iridophores (guanidine-rich pigmented cell); defects in pigment cell differentiation or chromatophore interactions cause abnormal pigment patterns (Kelsh et al. 2009; Parichy et al. 2000; Parichy et al. 1999; Iwashita et al. 2006; Rawls, Mellgren, and Johnson 2001; Singh and Nusslein-Volhard 2015). In wild-type fish, the zebrafish stripe pattern can generally be divided into embryonic, metamorphic and adult stages. The embryonic pattern is characterized by three sets of melanophore stripes along the dorsal and ventral myotomes, and the horizontal myoseptum. During metamorphosis, the growing xanthophore population is recruited to the myoseptum where they form the first adult interstripe as adult melanophores populate the surrounding area in the flanks to form the first and second adult stripes (Kelsh et al. 2009; Singh and Nusslein-Volhard 2015). At this time, embryonic melanophores caught in the developing interstripe are either repulsed by xanthophores and migrate to join the stripe, or become trapped and die in situ. The boundaries between stripes and interstripes become increasingly sharp, and the zebrafish transitions to the juvenile pattern of one interstripe with two bordering stripes.
around 28 days post fertilization (dpf). In adulthood, stripes and interstripes develop around this juvenile pattern to achieve the final striping pattern (Kelsh et al. 2009; Parichy and Spiewak 2015; Singh and Nusslein-Volhard 2015).

In previous studies, zebrafish pigment pattern mutants could be classified into two groups: fish with defects in the development of chromatophores, and fish with normal development of pigment cells but disrupted adult stripe patterns. Zebrafish mutants in the first class are utilized to interrogate the developmental pathways surrounding melanophore and melanoblast (melanophore progenitor) differentiation, maintenance, survival, proliferation and migration (Parichy and Spiewak 2015). Melanophores, along with peripheral neurons, glia and the other zebrafish chromatophores, xanthophores and iridophores, are all derived from the neural crest (Mort, Jackson, and Patton 2015; Parichy and Spiewak 2015). Notably, studies of a mutant fish called 'puma' revealed that early embryonic melanophores arise from melanogenic progenitor cells derived directly from the neural crest without a stem cell intermediate, and all subsequent melanophores arise from a melanophore stem cell (MSC) intermediate (Parichy and Turner 2003).

Zebrafish in this second category can be used to elucidate mechanisms for signaling pathways and cell-cell interactions involved in wild-type melanophore biology. Notably, the jaguar/obelix mutant, classified by wide stripes and indistinct borders, is defective in the process of intracellular aggregation and dispersion of the melanosome known as background adaptation (Iwashita et al. 2006). In melanophores, this process is mediated by activated PLC cleaving phosphatidylinositol 4,5-bisphosphate into IP3 and DAG which then signals through Ca$^{2+}$ influx to stimulate melanin translocation (Salim and A 2012; Vesely and Hadley 1979). Interestingly,
this process is mediated preferentially by GNAQ subunits downstream of adrenergic agonists for α-adrenergic receptors (Wu et al. 1992). Further interrogation into this mutant and others in this vein could elucidate positional and adrenergic signaling pathways in melanophores.

Therefore, utilizing zebrafish as a genetic tool, scientists have been able to study the role of genes in a variety of developmental pathways. Importantly, these pathways involved in differentiation, proliferation, survival, signaling and migration, are known to be potentially tumorigenic when de-regulated. With this in mind, we utilized well characterized zebrafish melanophore biology as a platform to understand how oncogenic expression of GNAQ/11 alters melanophore biology to facilitate tumorigenicity.
3.3. Results

3.3.1. Tg(mitfa:GNAQ/11Q209L) transgenic zebrafish display in vivo pigmentation defects throughout development

Based on previous observations of pigmentation defects in chimeric and adult transgenic animals (Chapter 2), we began a systematic analysis of pigmentation phenotypes in stable Tg(mitfa:GNAQ/11Q209L) transgenic lines. Melanophores in embryonic zebrafish are derived directly from the neural crest, whilst adult melanophores arise from an intermediate neural crest-derived stem cell population located by the dorsal root ganglia (Budi, Patterson, and Parichy 2011; Dooley et al. 2013). Thus, it was possible that the pigment defects of the GNAQ/11Q209L transgenics represent an adult phenotype that is absent from the embryos. To address this question, we generated cohorts of wild-type and transgenic (Tg+) clutchmates from the Tg(mitfa:GNA11Q209L) Q-1 line and followed them through development. As early as 3 days post fertilization (dpf), differences in pigmentation were apparent in the two genotypes (Figure 3.1A). At 3 dpf, the wild-type embryos had dorsal and ventral melanophore stripes in addition to an ordered lateral stripe across the horizontal myoseptum (Figure 3.1A), and by 3.5 dpf most melanophores had already migrated off the yolk sac to join the ventral stripe (Supplementary Video S3.1 and S3.2). In contrast, the Q-1 Tg+ zebrafish were hyperpigmented and possessed a disordered melanophore lateral stripe at 3 dpf and they clearly maintained melanophore localization over the yolk sac compared to Tg- clutchmates at 3.5 dpf (Figure 3.1A, Supp. Video S3.1 and S3.2). The pigment defects clearly persisted into metamorphosis, where at 14 dpf the Tg+ fish showed even higher levels of hyperpigmentation (Figure 3.1B), and had still failed to form ordered lateral stripes (Figure 3.1B, red box) compared to Tg- clutchmates.
By 6 weeks post fertilization, wild-type zebrafish have the foundations of the adult zebrafish stripe pattern. The defects apparent in embryonic and metamorphic Q-1 Tg^+ phenotypes persist and worsen as the fish enter adulthood and develop more stripes (Figure 2.2, Figure 3.1C). In addition to hyperpigmentation and non-linear stripe patterns, Tg^+ adult zebrafish have

Figure 3.1. Tg(mifia:GNAQQ209L) zebrafish display pigmentation defects throughout development. (A) At 3.5 dpf, line Q-1 Tg^+ embryonic fish display hyperpigmentation, mislocalization and boundary phenotypes compared to wild-type controls. (B) Q-1 Tg^+ metamorphic fish at 14 dpf are hyperpigmented and melanophores fail to adhere to the normal lateral stripe along the horizontal myoseptum (red box, b'). Magnified inset of the flanks of the metamorphic fish (b'', purple box) shows ordered stripe (red arrow) in the wild-type fish and disordered melanophores across the flanks in Q-1 Tg^+ fish. (C) At 6 weeks post fertilization juvenile Q-1 Tg^+ fish are hyperpigmented and fail to establish linear stripes compared to controls.
mislocalized melanophores in the interstripe region compared to non-transgenic clutchmates (Figure 3.2A,B). This mislocalization is not limited to the interstripe region, as we previously detected pigment at aberrant, internal locations within adult $Tg^+$ fish (Chapter 2, Figure 2.2). Finally, these images also reveal a difference in melanin dispersion in $Tg^+$ compared to $Tg^-$ melanophores under the same lighting conditions (Figure 3.2C). Specifically, the melanin appeared punctate and aggregated in the $Tg^-$ melanophores, but it has a highly dendritic phenotype in the $Tg^+$ clutchmates. Importantly, we also analyzed a second line, called 11-1, that carried the $Tg(mita:GNA11Q29L)$ and observed the same spectrum of pigment defects and the same time of onset (Supplementary Figure S3.3 and data not shown). Taken together, these data show that expression of oncogenic GNAQ/11 disrupts melanophore biology throughout development in $Tg^+$ zebrafish.

3.3.2. Background adaptation assays allow for quantitation of pigmentation defects in $Tg(mita:GNAQ^{O29L})$ zebrafish

Background adaptation is a central property of melanophores that begins in zebrafish around 4 dpf (Logan, Burn, and Jackson 2006). Given our finding that wild-type and $Tg^+$ melanophores have distinct morphologies under similar background conditions (Figure 3.2C), we wanted to directly assess the response of $Tg^+$ melanophores for background adaptation. For this, we generated $Tg^+$ and $Tg^-$ clutchmates, placed them on light or dark backgrounds at 4 dpf and allowed them to adapt before euthanasia and analysis at 5 dpf (Figure 3.3A). We found that both $Tg^+$ and $Tg^-$ embryonic zebrafish expand and contract melanin within their melanophores appropriately according to background (3.3A,B). Interestingly, it was previously posited that GNAQ/11 had a role in the adrenergic pathway which contributes to the melanin translocation
Figure 3.2. Melanophores mislocalize to the interstripe region in Tg(mitfa:GNAOQ0209L) zebrafish. (A) By 6 weeks post fertilization the interstripe (red arrow) in Tg- animals is completely clear of melanophores. (B) Tg+ clutchmates have a defined, but highly non-linear interstripe (red arrow), which still contains mislocalized melanophores (white arrows). (C) Magnified view of the sparser melanophores above the first stripe shows differences in melanin aggregation states between Tg+ and Tg- controls under the same lighting conditions.
response in advance of the slower hormonal pathways (Salim and A 2012; Wu et al. 1992). Thus, we examined the response to background adaptation over a shorter timescale, we found that \( Tg^+ \) melanophores responded to epinephrine stimulation with a larger amplitude of response and slight shift in melanin aggregation kinetics (Supplementary Figure S3.4). Thus, these data suggest that oncogenic GNAQ plays a role in the initial melanin translocation response.

We were also able to exploit these background adaptation assays to visualize, and directly quantify, additional melanophore phenotypes in \( Tg^+ \) embryonic zebrafish. First, we assessed the degree of hyperpigmentation in the 5 dpf background adapted embryonic fish and showed that \( Tg^+ \) zebrafish had a higher percentage of total area covered in melanin, compared to \( Tg^+ \) clutchmate controls, under both light and dark background conditions (Figure 3.3B; \( p<0.0001 \)). Notably, this increased coverage resulted from two distinct changes. First, there was a substantial increase in the area of melanin coverage per melanophore in both dark (Figure 3.3C; \( p<0.0001 \)) and light (Figure 3.3D) adapted \( Tg^+ \) fish. Second, the \( Tg^+ \) fish had significantly more melanophores, as judged by quantification of melanin puncta in the light adapted fish (Figure 3.3E; \( p<0.0001 \)). Importantly, these phenotypes were all \( tp53 \)-independent (Supplementary Figure S3.5). These findings show that oncogenic GNAQ disrupts multiple aspects of melanophore biology.

**3.3.3. \( Tg(\text{mitfa}:\text{GNAQ}^{209L}) \) expression increases melanin distribution without increasing melanophore size**

We wondered whether the observed increase in melanin coverage per cell was related to alterations in the background adaptation response, or represented an unrelated change. Thus, to
Figure 3.3. Background adaptation assays reveal changes in melanin coverage and melanophore number in Tg(mifia:GNAQ\textsuperscript{Q205L}) zebrafish. (A) Tg\textsuperscript{+} and Tg\textsuperscript{-} zebrafish were background adapted from 4-5 dpf. (B) Tg\textsuperscript{+} embryonic fish display greater melanin coverage than Tg\textsuperscript{-} clutchmate controls regardless of background though both genotypes expand and contract melanin properly over dark/light conditions. (C) Area of melanin coverage is greater in Tg\textsuperscript{+} expressing melanophores. (D) Melanophores (red circles) are highlighted in the ventral side of light adapted 5 dpf zebrafish. (E) Tg\textsuperscript{+} zebrafish have more melanophores than Tg\textsuperscript{-} controls at 5 dpf. (F) The lateral view across the yolk sac of 3 dpf fish highlighting differences in pigmented area upon Tg\textsuperscript{+} expression. (G) Graphical representation of area of melanin dispersion in Tg\textsuperscript{+} versus Tg\textsuperscript{-} melanophores across 3 dpf yolk sac.
precede the onset of background adaptation capabilities at 4 dpf (Logan, Burn, and Jackson 2006), we quantified the area of melanin coverage within the yolk sac melanophores at 3 dpf (Figure 3.3F). This showed that the melanin coverage per cell was significantly increased, compared to Tg- clutchmate controls (Figure 3.3G; p<0.0001). Importantly, this was similar to the increases at later timepoints, suggesting that it reflects an altered basal state that is independent of background adaptation response.

These in vivo analyses clearly demonstrated that oncogenic GNAQ expands the per cell melanin coverage, but yielded no information about cell size. To address this question, we

Figure 3.4. Tg(mitfa:GNAQ^{Q209L}) expressing melanophores have increased melanin dispersion in vitro without increasing cell size. (A) Pigmented area per cell in Tg+ melanophores versus Tg- controls after purification from adult fins and plating and analysis in vitro. (B) Histogram representation (b') of melanophore size in Tg+ and Tg- populations as measured by volume displacement and bar graph representation (b'') of the mean (Tg-=1,184fL, Tg+=1,131fL) and standard deviations (Tg-=579, Tg+=623) in these populations.
adapted established protocols (Higdon, Mitra, and Johnson 2013; Yamanaka and Kondo 2014) to purify melanophores for in vitro analysis. Initially, we purified cells from $Tg^-$ versus $Tg^+$ adult fins, which show the same melanophore phenotypes as the skin (Supplemental Figure S3.6). After plating the cells in 2D we confirmed that the $Tg^+$ melanophores maintained the expanded per cell melanin distribution phenotype (Figure 3.4A). Next, we purified cells from $Tg^-$ versus $Tg^+$ embryos and assayed cell size by measuring mean volume displacement using a Coulter counter. The $Tg^+$ (n=14,272) and $Tg^-$ (n=17,775) melanophores showed a highly similar distribution of sizes (Figure 3.4B), with the $Tg^+$ cells showing a slight shift towards smaller mean sizes ($Tg^+$ mean=1,131fL versus $Tg^-$ mean=1,184fL) that was significantly significant because of the large sample size. Together, these data shown that oncogenic GNAQ expands the per cell melanin coverage without increasing cell size.

3.3.4. $Tg$(*mitfa*:GNAQ$^{Q209L}$) expression in melanophores results in increased dendricity and altered morphology

We also examined the properties of $Tg^-$ versus $Tg^+$ melanophores that had been plated in vitro following isolation from adult fins. Consistent with the in vivo phenotype (Figure 3.2C), the $Tg^+$ melanophores appeared highly dendritic compared with $Tg^-$ controls (Figure 3.5A). To quantify this dendricity, we calculated cell convexity by taking the ratio of the cell perimeter to convex hull perimeter (Figure 3.5B). This established a highly significant difference ($p<0.0001$), with both the mean and minimal convexity being considerably lower in $Tg^+$ melanophores compared to the $Tg^-$ controls.

We wondered whether the morphology of the cells would change over time. Thus, we plated the cells on collagen, as previously described (Yamanaka 2014), and then followed their
behavior over time by conducting time-lapse video microscopy. The resulting time-lapse images demonstrated that the $Tg^+$ melanophores were highly dynamic (see Figure 3.5C for representative), and capable of switching back and forth from a highly dendritic to more rounded states. In contrast, $Tg^-$ melanophores did not display these stunning changes in cell morphology but rather appeared to expand and contract their melanin within their cell boundaries (Supplementary Figure S3.7).

We then extended these studies to compare the motility of the $Tg^-$ versus $Tg^+$ melanophores. For these studies, isolated melanophores were plated in serum-free media and one day later switched to media containing 10% FBS before conducting time-lapse imaging for either 24 or 36 hours. These experiments revealed striking differences in the behavior of $Tg^-$ versus $Tg^+$ melanophores (Figure 3.6A-C, Supplemental Videos S3.8 and S3.9). Colored arrows highlight representative individual $Tg^-$ and $Tg^+$ melanophores at 0, 12, 24, and 36 hour timepoints (Figure 3.6A). To understand the precise nature of these changes, we marked the migratory tracks of individual $Tg^-$ and $Tg^+$ melanophores over a 24 hour period (Figure 3.6B). Quantitation of the total distance covered established that $Tg^+$ melanophores migrate over significantly longer distances than their $Tg^-$ control counterparts (Figure 3.6C; p<0.0001). However, despite this enhanced migratory ability, the $Tg^+$ melanophores display reduced persistence compared to $Tg^-$ controls (Figure 3.6D; p<0.017). Persistence was calculated as the ratio of the distance between the location of the melanophore at the first and last timepoint and the total distance traveled over the 24 hour period. Taken together, these results show a defect in motility in melanophores upon $Tg^+$ expression.
Figure 3.5. \textit{Tg(mitfa:GNAQ209L)} expressing melanophores have a more dendritic phenotype than controls. (A) Representative images of \textit{Tg} and \textit{Tg}\textsuperscript{+} melanophores isolated from fins of adult animals and plated \textit{in vitro} on collagen (a') and magnified images demonstrating dendricity (a''). Scale bar = 100\,\mu m. (B) Quantitation of \textit{Tg} and \textit{Tg}\textsuperscript{+} melanophore dendricity \textit{in vitro} as measured by convexity. (C) Time-lapse images capture the change in morphology of one \textit{Tg}\textsuperscript{+} melanophore from 0:00 to 12:00 hours of imaging. Scale bar = 25\,\mu m, time of the image is denoted in the upper left hand corner.
Figure 3.6. *Tg(mita:GNAQQ209L)* expressing melanophores are highly motile but lack persistence in vitro. Melanophores were isolated from adult zebrafish fins, plated on collagen in the serum-free media, and then serum added before time-lapse video microscopy was conducted. (A) Representative 10X images of *Tg* (top) and *Tg*+ (bottom) melanophores at 12 hour intervals. Arrows (purple, blue, green, pink, yellow, orange) are color coordinated across time-lapse stills to highlight a single melanophore in the field over time. (B) Representative plot of tracks for one field of *Tg* and *Tg*+ in vitro melanophores migrating over 24 hours. *Tg* n= 10, *Tg*+ n=13. (C) Quantitation of total distance covered over 24 hours. (D) Quantitation of persistence over 24 hours.
3.3.5. *Tg(mita:GNAQ<sup>Q209L</sup>) expressing melanophores have increased survival capacity*

It is well established that normal melanophores undergo apoptosis if they persist in the interstripe regions. Since *Tg*<sup>+</sup> melanophores are consistently detected within interstripe regions (Figure 3.2), we hypothesized that oncogenic GNAQ signaling was promoting their survival. To address this possibility, we directly compared the survival of *Tg*<sup>-</sup> and *Tg*<sup>+</sup> melanophores isolated from fish fins and plated on collagen coated 96-well culture dishes. As melanophores are heavily pigmented, we could not follow cell number by traditional trypan-blue exclusion assays. Therefore, we counted the live cells present in 4 random fields per well from Day 1 to up to Day 7 post-seeding (n=2-4 wells/day/genotype). For each well, the counts from the four fields were averaged, and normalized to the Day 1 counts. Importantly, the *Tg*<sup>-</sup> and *Tg*<sup>+</sup> melanophores never proliferate in culture, based on our time-lapse imaging studies (data not shown). Thus, the observed cell number directly reflects *in vitro* survival. The two genotypes show similar survival kinetics from Day 1 to Day 2, but after this initial loss, the *Tg*<sup>+</sup> melanophores showed improved *in vitro* survival compared to the *Tg*<sup>-</sup> controls (Figure 3.7A). This enhanced survival was particularly apparent at later timepoints (Figure 3.7A,B). Thus, transgene expression enhances melanophore survival in the *in vitro* setting.

To assess survival in the *in vivo* setting, we decided to follow *Tg*<sup>-</sup> and *Tg*<sup>+</sup> clutchmate fish through the process of metamorphosis. In normal animals, as the zebrafish transitions from embryonic to adult striping, the xanthophores begin to dominate the embryonic stripe region along the horizontal myospetum while metamorphic melanophores populate the ventral
Figure 3.7. \textit{Tg\textit{(mif}\textit{a}:\textit{GNAQ}^{\textit{Q}209L}) expressing melanophores have increased cell viability \textit{in vitro}.} (A) Graphical representation of melanophore survival \textit{in vitro}, p-value close to zero by GEE with link function of Poisson distribution. n=4 wells for days 1, 2, 3, and 4; n=2 wells for days 5 and 7. (B) Representative images of \textit{Tg} and \textit{Tg}\textsuperscript{+} melanophores plated \textit{in vitro} over the course of 1, 5 and 10 days depicting melanophore survival. Images taken at 10x.
myotomes (Kelsh et al. 2009). The embryonic and early metamorphic melanophores can persist for a time in the developing xanthophore interstripe, but are ultimately forced to move into the early adult stripes or to die in situ by the repulsive cues of the xanthophore stripe. We conducted careful observation and quantitation of $Tg^+$ and $Tg^-$ zebrafish through metamorphosis and into adulthood (14dpf-44dpf; Figure 3.8). This revealed increased numbers of melanophores in the xanthophore interstripe of $Tg^+$ fish at all time points, with significant differences in melanophore number beginning at 18 dpf (Figure 3.8A,C; p<0.01). Given the persistent survival of numerous $Tg^+$ melanophores in the interstripe regions, we conclude that oncogenic GNAQ alters two distinct aspects of melanophore patterning. First, it abrogates the melanophore response to the repulsive cues of the xanthophores that normally induces exit from the interstripe region. Second, it allows these interstripe-occupying melanophores to escape their normal apoptotic fate. Finally, in addition to uncovering stripe patterning defects, our analysis of metamorphic stages shows that the $Tg^+$ fish have a higher total number of melanophores than the $Tg^-$ controls, especially towards the end of this process (Figure 3.8A). While the apoptosis defect clearly contributes to this accumulation, the presence of excess melanophores within the flanks of the zebrafish strengthens the possibility that enhanced proliferation is also occurring.

3.3.6. Analysis of differential expression patterns of transgenic versus non-transgenic melanophore populations

To understand the molecular bases of the observed melanophore defects, we wished to conducted gene expression analyses of $Tg^-$ versus $Tg^+$ melanophores. Unfortunately, the protocol
Figure 3.8. *Tg(mita:GNAQQ209L)* expressing embryonic melanophores persist in the interstripe of zebrafish undergoing the metamorphic to adult stripe patterning transition. (A) Pigment pattern throughout metamorphosis in *Tg* and *Tg*+ zebrafish, numbers indicate dpf. *Tg* zebrafish gradually increase melanophore number along the middle of each ventral myotome which merge to form the first two stripes around the xanthophore interstripe along the horizontal myoseptum. As stripes form, embryonic melanophores located along the myoseptum either apoptose or migrate from the interstripe region. At corresponding stages, *Tg*+ zebrafish quickly populate their flanks with more melanophores than *Tg* clutches without forming stripes more quickly. Embryonic melanophores in the *Tg*+ interstripe persist into the adult stages indicating a failure to apoptose. Scale bar = 1mm. (B) Enhanced image of 44dpf *Tg* (left) and *Tg*+ (right) interstripe. *Tg*+ zebrafish have melanophores populating the interstripe as compared to *Tg* clutchmate controls. Scale bar = 500μm. (C) Quantitation of melanophores in the interstripe in *Tg* and *Tg*+ fish through different stage in metamorphosis. n=20 fish. Unpaired T-test run for every data point in Prism. ***p-value<0.0001, **p>0.01, *p>0.05.
that we used to generate melanophore populations for 2D culture was insufficient for this goal; the resulting cell preparation may contain other chromatophores and produces low yield for RNA preparations (data not shown). Thus, by combining and adapting steps from several reported protocols (Higdon, Mitra, and Johnson 2013; Yamanaka and Kondo 2014), we developed an optimized procedure for isolating melanophores for RNA analyses. This was conducted using 5 dpf embryos, which display all of the known in vivo $Tg^+$ melanophore phenotypes (Figure 3.1). As embryos contain about 300-400 melanophores (Kimmel et al. 1995), we conducted our preparations using pools of 400-1000 embryos. The method involves a brief trypsinization to triturate the zebrafish eyes away from the bodies, thereby preventing the melanized cells of the retinal pigmented epithelium (RPE) from contaminating the cutaneous melanophores. The eye-free larvae are then enzymatically dissociated into a single cell suspension, filtered to remove large contaminants, and enriched for melanophores using two different density-dependent centrifugations (Figure 3.9). The other pigment containing cells in zebrafish, namely the guanine-rich iridophores and pteridine-containing xanthophores, are mostly removed by this method and represent only $\sim 1\%$ of the final purified melanophore preparations (data not shown).

Using this melanophore isolation method, we generated purified populations of roughly 50,000-140,000 melanophores from cohorts of $Tg^+$ and $Tg^-$ embryonic zebrafish in triplicate. RNA was then isolated and libraries for transcriptome sequencing were generated. Following sequence analysis and mapping, the data were filtered to yield 17,121 expressed protein-coding genes to be used for further study. First, we performed unsupervised clustering on these data to determine whether our $Tg^-$ and $Tg^+$ melanophore populations could be differentiated in an
Remove anal and tail fins

Separate eyes from larva

Dissociate into single cell suspension

Density dependent centrifugations

Cells for RNAseq libraries

**Figure 3.9. Schematic of purification method for melanophores and subsequent preparation for RNAseq.** (A) The tail and anal fins of adults Tg⁺/Tg zebrafish are dissected or Tg⁺/Tg⁻ zebrafish are raised to 5 dpf and briefly trypsinized to remove the eyes. (B) Fin or larva tissue is dissociated to a single cell suspension and pigmented melanophores (black arrows) are visible among many other cell types and debris, including the occasional xanthophore (yellow circle). The cell suspension is placed on top of a Percoll gradient and centrifuged to generate (C) a purified population of melanophores suitable for either *in vitro* experiments in the case of the fin dissection, or RNAseq in the case of the embryonic fish.
unbiased fashion. Gratifyingly, the $Tg$ and $Tg^+$ melanophore populations form two separate clades indicating reproducible gene expression changes in response to $Tg^+$ expression (Figure 3.10A). In addition, expression in both melanophore populations correlated with previous expression analyses of ~5 dpf melanophore samples (Higdon, Mitra, and Johnson 2013), indicating that both $Tg$ and $Tg^+$ populations maintain a general melanophore expression signature (data not shown).

Our first aim was to use the RNAseq data to identify genes that were differentially expressed between $Tg$ and $Tg^+$ melanophore populations. To this end, differential expression was tested using DESeq (Anders and Huber, 2010) and significantly different genes were defined as those having a log2 fold change $\geq 1$ with an adjusted p-value $\leq 0.05$. These thresholds produced a list of 353 differentially expressed genes (DEGs) between $Tg$ and $Tg^+$ melanophores. Of these DEGs, 206 genes were up-regulated and 147 genes were down-regulated upon $Tg^+$ expression (Figure 3.10B, data not shown). We focused on genes that had clear human orthologs and/or some functional annotation in zebrafish. Human ortholog mapping was based on positional information and BLAST alignments (personal communication, Adam Amsterdam and Sebastian Hoersch). Because of an ancient whole genome duplication event, many zebrafish genes have at least 2 orthologs of mammalian genes (Taylor et al. 2001). To prevent redundant gene symbols for further work and functional analysis, when more than one zebrafish gene shared a human ortholog we selected the zebrafish gene that was differentially expressed or, if neither showed differential expression, the gene with the highest average expression (Supplementary Figure S3.10). Moving forward in this fashion, of the original 353 DEGs, 143 up-regulated and 109 down-regulated genes upon $Tg^+$ expression mapped to human orthologs.
Figure 3.10. Unsupervised clustering of Tg- and Tg(mitfa:GNAQQ209L) melanophore populations. (A) Dendrogram and heatmap of unsupervised clustering of 17,121 protein coding genes expressed in Tg-/Tg' melanophore populations. Clustering (Ward’s Method) was done using TIBCO Spotfire. (B) Heatmap of 353 differentially expressed genes between Tg-/Tg' melanophore populations, Log2FC>1, p-adj<0.05.

The top 25 DEGs enriched and top 20 DEGs depleted in Tg' expressing melanophores are summarized (Table 3.1 and Table 3.2).

These gene sets contain members of diverse biological functions, which we further interrogated using both GeneGo and DAVID functional analysis tools. Interesting categories that consistently were revealed as a result of these analyses included axon guidance and cytoskeletal binding (Supplemental Figure S3.11). While these results are exciting, we experienced difficulty in attaining significant adjusted p-values. Regardless, genes in these categories could help
elucidate if oncogenic GNAQ/11 signaling plays a role in switching the melanophore to a more neuronal-like program or help establish the mechanism by which cells increase motility upon Tg+ signaling.

To further assess the functional consequences of Tg+ expression in melanophores, we used gene set enrichment analysis (GSEA) and human gene sets provided by MSigDB in the canonical pathways group. GSEA revealed that Tg+ melanophore populations are enriched in categories associated with cell cycle regulation (Figure 3.11). This is consistent with our hypothesis that the expanded number of Tg+ melanophores in vivo (Figure 3.8) result in part from increases in proliferation. Additionally, GSEA revealed that the Tg- class are enriched in cell adhesion and extracellular matrix gene sets (Figure 3.12A). We speculate that perturbations of these activities could contribute to the altered morphology and migration properties of the Tg+ melanophores.

### Table 3.1. Top 25 Differentially Expressed genes enriched in melanophores upon Tg+ Expression

<table>
<thead>
<tr>
<th>Zebrafish Symbol</th>
<th>Human Symbol</th>
<th>Tg+</th>
<th>Tg-</th>
<th>Log2FC</th>
<th>P-val</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>dctpp1</td>
<td>DCTPP1</td>
<td>0.019</td>
<td>1.618</td>
<td>6.386</td>
<td>8.980E-06</td>
<td>Pyrimidine metabolism</td>
</tr>
<tr>
<td>grn2</td>
<td>GRN</td>
<td>0.695</td>
<td>5.718</td>
<td>6.047</td>
<td>4.011E-15</td>
<td>Signal transduction</td>
</tr>
<tr>
<td>grn1</td>
<td>GRN</td>
<td>3.306</td>
<td>9.297</td>
<td>5.845</td>
<td>1.397E-14</td>
<td>GRN paralogue</td>
</tr>
<tr>
<td>lrn2</td>
<td>LRRN2</td>
<td>0.221</td>
<td>2.785</td>
<td>5.287</td>
<td>8.542E-35</td>
<td>Neuronal signal transduction, membrane</td>
</tr>
<tr>
<td>ill4</td>
<td>CLEC4E</td>
<td>0.092</td>
<td>1.359</td>
<td>4.547</td>
<td>1.981E-02</td>
<td>Immune system signaling</td>
</tr>
<tr>
<td>crhbp</td>
<td>CRHBP</td>
<td>2.668</td>
<td>6.456</td>
<td>4.036</td>
<td>8.192E-06</td>
<td>Corticotropin-releasing hormone binding</td>
</tr>
<tr>
<td>npdc1b</td>
<td>NPDC1</td>
<td>0.796</td>
<td>3.559</td>
<td>3.989</td>
<td>1.114E-04</td>
<td>Neuroproliferation</td>
</tr>
<tr>
<td>sir/dkey-188/13.9</td>
<td>IF27L2</td>
<td>0.600</td>
<td>3.102</td>
<td>3.930</td>
<td>9.028E-07</td>
<td>Integral component of membrane</td>
</tr>
<tr>
<td>sed</td>
<td>SCDS</td>
<td>0.133</td>
<td>1.133</td>
<td>3.658</td>
<td>1.797E-02</td>
<td>Metal-ion binding</td>
</tr>
<tr>
<td>necab1</td>
<td>NECAB1</td>
<td>0.308</td>
<td>1.883</td>
<td>3.600</td>
<td>2.555E-03</td>
<td>Neuronal calcium binding</td>
</tr>
<tr>
<td>reep1</td>
<td>REEP1</td>
<td>0.260</td>
<td>1.737</td>
<td>3.518</td>
<td>3.212E-04</td>
<td>Microtubule binding</td>
</tr>
<tr>
<td>rds</td>
<td>RDH16</td>
<td>4.047</td>
<td>7.534</td>
<td>3.437</td>
<td>1.866E-12</td>
<td>Oxidoreductase activity</td>
</tr>
<tr>
<td>gbx2</td>
<td>GBK2</td>
<td>1.369</td>
<td>4.107</td>
<td>3.317</td>
<td>1.182E-20</td>
<td>Nervous system development</td>
</tr>
<tr>
<td>ano1</td>
<td>ANO1</td>
<td>1.197</td>
<td>3.766</td>
<td>3.290</td>
<td>1.577E-20</td>
<td>Calcium activated cation channel activity</td>
</tr>
<tr>
<td>phactr3a</td>
<td>PHACTR3</td>
<td>1.292</td>
<td>3.852</td>
<td>3.212</td>
<td>4.615E-03</td>
<td>Phosphatase and actin regulator</td>
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<tr>
<td>ccdd191</td>
<td>KIAA1407</td>
<td>0.937</td>
<td>3.155</td>
<td>3.095</td>
<td>5.880E-18</td>
<td>Uncharacterized</td>
</tr>
<tr>
<td>tmem200a</td>
<td>TMEM200A</td>
<td>0.230</td>
<td>1.241</td>
<td>3.043</td>
<td>4.878E-03</td>
<td>Integral component of membrane</td>
</tr>
<tr>
<td>fsd1</td>
<td>FSD1</td>
<td>0.189</td>
<td>1.051</td>
<td>2.987</td>
<td>2.163E-02</td>
<td>Negative regulation of neuronal apoptosis</td>
</tr>
<tr>
<td>chst8</td>
<td>CHST8</td>
<td>0.216</td>
<td>1.096</td>
<td>2.869</td>
<td>4.397E-02</td>
<td>Sulfotransferase activity</td>
</tr>
<tr>
<td>crpl2</td>
<td>CRPL2</td>
<td>4.535</td>
<td>7.427</td>
<td>2.828</td>
<td>5.924E-07</td>
<td>Cell proliferation</td>
</tr>
<tr>
<td>rab3c</td>
<td>RAB3C</td>
<td>0.361</td>
<td>1.559</td>
<td>2.791</td>
<td>2.514E-02</td>
<td>GTPase mediated signal transduction</td>
</tr>
<tr>
<td>btr31</td>
<td>BTR31</td>
<td>0.545</td>
<td>1.926</td>
<td>2.614</td>
<td>1.433E-02</td>
<td>Integral component of membrane</td>
</tr>
<tr>
<td>maplt1b</td>
<td>MAPLT1</td>
<td>0.906</td>
<td>2.802</td>
<td>2.608</td>
<td>1.739E-07</td>
<td>Microtubule binding</td>
</tr>
<tr>
<td>alcama</td>
<td>ALCAMA</td>
<td>3.417</td>
<td>5.969</td>
<td>2.607</td>
<td>2.627E-10</td>
<td>Axon extension</td>
</tr>
<tr>
<td>fot1a</td>
<td>FOT1A</td>
<td>0.871</td>
<td>2.513</td>
<td>2.549</td>
<td>2.904E-05</td>
<td>TGF-beta signaling pathway</td>
</tr>
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Table 3.2. Top 20 Differentially Expressed genes depleted in melanophores upon Tg+ Expression

<table>
<thead>
<tr>
<th>Zebrafish Symbol</th>
<th>Human Symbol</th>
<th>Tg-</th>
<th>Tg+</th>
<th>Log2FC</th>
<th>p-ad</th>
<th>Notes</th>
</tr>
</thead>
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<tr>
<td>CR956626.1</td>
<td>ADGRG7</td>
<td>1.573</td>
<td>0.100</td>
<td>-5.244</td>
<td>2.069E-02</td>
<td>Adhesion G-protein coupled receptor</td>
</tr>
<tr>
<td>grap2b</td>
<td>GRAP2</td>
<td>2.548</td>
<td>0.533</td>
<td>-3.353</td>
<td>2.084E-11</td>
<td>Signaling by GPCR</td>
</tr>
<tr>
<td>dok6</td>
<td>DOK6</td>
<td>2.071</td>
<td>0.410</td>
<td>-3.247</td>
<td>1.759E-04</td>
<td>Ret-mediated neurite outgrowth</td>
</tr>
<tr>
<td>irx4b</td>
<td>IRX4</td>
<td>5.501</td>
<td>2.569</td>
<td>-3.137</td>
<td>1.988E-07</td>
<td>Regulation of transcription</td>
</tr>
<tr>
<td>ftln7</td>
<td>FBLN7</td>
<td>2.818</td>
<td>0.766</td>
<td>-3.049</td>
<td>3.345E-13</td>
<td>Cell adhesion</td>
</tr>
<tr>
<td>fyb (1 of 2)</td>
<td>FYB</td>
<td>3.081</td>
<td>1.051</td>
<td>-3.015</td>
<td>9.324E-07</td>
<td>Immune signal transduction</td>
</tr>
<tr>
<td>nr4a2b</td>
<td>NRR4A2</td>
<td>6.306</td>
<td>3.534</td>
<td>-2.866</td>
<td>4.799E-07</td>
<td>Canonical Wnt signaling pathway</td>
</tr>
<tr>
<td>ankrd35db</td>
<td>ANKRD34B</td>
<td>1.872</td>
<td>0.453</td>
<td>-2.845</td>
<td>6.277E-04</td>
<td>Uncharacterized</td>
</tr>
<tr>
<td>opn3</td>
<td>OPN3</td>
<td>3.723</td>
<td>1.440</td>
<td>-2.802</td>
<td>2.651E-07</td>
<td>G-protein coupled photoreceptor activity</td>
</tr>
<tr>
<td>pel1a</td>
<td>PEL1</td>
<td>2.631</td>
<td>0.862</td>
<td>-2.654</td>
<td>4.878E-03</td>
<td>Ubiquitin protein ligase activity, NF-kB signaling</td>
</tr>
<tr>
<td>stich211-76m11.3</td>
<td>CD244</td>
<td>1.687</td>
<td>0.36</td>
<td>-2.588</td>
<td>9.772E-05</td>
<td>ATP-binding</td>
</tr>
<tr>
<td>cldn5b</td>
<td>CLDN5</td>
<td>1.522</td>
<td>0.391</td>
<td>-2.586</td>
<td>1.556E-02</td>
<td>Calcium independent cell-cell adhesion</td>
</tr>
<tr>
<td>b3gnt3</td>
<td>B3GNT3</td>
<td>3.095</td>
<td>1.194</td>
<td>-2.540</td>
<td>1.457E-05</td>
<td>Carbohydrate metabolic process</td>
</tr>
<tr>
<td>chaddb</td>
<td>CHADD</td>
<td>2.324</td>
<td>0.779</td>
<td>-2.417</td>
<td>4.527E-03</td>
<td>Collagen binding</td>
</tr>
<tr>
<td>fam1b8b2</td>
<td>FAM1B8B2</td>
<td>1.684</td>
<td>0.513</td>
<td>-2.304</td>
<td>2.244E-02</td>
<td>Uncharacterized</td>
</tr>
<tr>
<td>arsg</td>
<td>ARSG</td>
<td>1.339</td>
<td>0.393</td>
<td>-2.285</td>
<td>4.045E-02</td>
<td>Uncharacterized</td>
</tr>
<tr>
<td>fmodb</td>
<td>FMOD</td>
<td>3.146</td>
<td>1.347</td>
<td>-2.246</td>
<td>1.099E-04</td>
<td>Extracellular matrix organization</td>
</tr>
<tr>
<td>mmp19</td>
<td>MMP19</td>
<td>2.555</td>
<td>1.027</td>
<td>-2.207</td>
<td>2.167E-04</td>
<td>Angiogenesis, extracellular matrix disassembly</td>
</tr>
<tr>
<td>kcng1</td>
<td>KCNG1</td>
<td>2.341</td>
<td>0.894</td>
<td>-2.203</td>
<td>4.107E-04</td>
<td>Ion transport</td>
</tr>
</tbody>
</table>

Figure 3.11. GSEA enrichment plots for enrichment upon Tg+ expression. Gene set enrichment plots of pathways enriched in Tg+ melanophore populations include the E2F hallmark targets, MCM pathway and Mitotic M/M/G1 Phase Reactome. Tg+ enrichment is on the left side of the plot (red).
Figure 3.12. GSEA enrichment plots for enrichment in Tg- melanophore populations. (A) Gene set enrichment plots of pathways enriched in Tg- melanophore populations include core matrisome, cell adhesion molecules, and integrin pathways. Tg- enrichment is on the right side of the plot (blue). (B) Heatmap of the top 19 genes driving enrichment in Tg- melanophore populations.
3.4. Discussion

For decades, zebrafish biologists have utilized genetic mutants to study the zebrafish stripe pattern thereby providing fundamental insights into melanophore development, signaling and differentiation. Our study is the first to extensively characterize the effects of an oncogene on stripe patterning. The resulting findings provide key insight into how expression of oncogenic GNAQ/IIQ^{209L}, a key initiating event in uveal melanoma development, alters the biological properties of melanophores prior to acquisition of transformed state. Our data show that oncogenic GNAQ/IIQ^{209L} induces dramatic pigmentation defects including hyperpigmentation, aberrant morphology and a failure to adhere to boundary cues that results in disorganized stripes and melanophore mislocalization. Through combined in vivo and in vitro approaches, we show that oncogenic GNAQ/IIQ^{209L} is driving changes in both internal signaling and the response to extracellular cues, and it alters a diverse range of biological processes including cell morphology, cell motility, cell survival and cell proliferation. Many of these changes are potentially pro-tumorigenic.

Our data show that the full range of pigment defects is apparent in Tg^+ zebrafish by 3 dpf and these continue and become even more exacerbated throughout development. It has previously been reported that embryonic larval melanophores and adult melanophores arise from distinct stem cell populations (Mort, Jackson, and Patton 2015; Parichy and Spiewak 2015; Singh and Nusslein-Volhard 2015; Budi, Patterson, and Parichy 2011). However, the consistency of the Tg^+ phenotypes across all developmental stages, and also between cutaneous and fin patterns, suggests that the effects of oncogenic GNAQ/11 signaling affects all melanophore stem cell populations regardless of their differing origins. This result is
not unexpected, as the mitfa-promoter driving the transgene functions early in melanophore
determination; nevertheless, this observation is key in demonstrating that experiments performed
in melanophores from both embryonic and adult animals are broadly informative.

Previous studies have suggested that GNAQ/11 signaling is involved in the regulation of
pigment dispersion and contraction (Salim and A. 2012; Wu et al. 1992). Our data show that the
background adaptation response persists in Tg+ fish but, consistent with the prior hypothesis, the
kinetics of this response are altered. Moreover, we found that the melanin is distributed over a
wider area of the cell in the Tg+ fish, compared to their wild-type controls, under both dark
(maximal expansion) and light (maximal contraction) background conditions. Notably, this
melanin expansion is observed at developmental stages that precede the emergence of
background adaption capabilities. Moreover, it is not associated with any increase in cell size.
Thus, it appears to reflect a fundamental change in the basal state of the melanosome. Our gene
expression analyses showed no significant differences in the levels of mRNA involved in
melanin biosynthesis between Tg− and Tg+ melanophores, suggesting that this is not simply due
to increase in the cell’s melanin content. Instead, we favor the notion that the observed increase
in melanin area reflects a relaxation of the melanosomes and/or is somehow related to changes in
cell topology.

We raise the possibility of topological changes above because our in vivo and in vitro data
unequivocally show that GNAQ209L signaling causes profound changes in both the morphology
and migratory properties of melanophores. Specifically, we see the Tg+ melanocytes adopt a
more dendritic phenotype in vitro, and they become more motile but lack persistence. To our
knowledge, this is the first time that oncogenic GNAQ has been shown to disrupt these

111
biological processes. Interestingly, Feng and colleagues have previously shown that oncogenic GNAQ/11 signaling promotes Yap nuclear translocation via RhoA and Rac1, which are well known regulators of the actin cytoskeleton and motility (Feng et al. 2014). Thus, it is tempting to speculate that activation of Rho/Rac by oncogenic GNAQ would also promote cell motility. Obviously, it remains to be determined whether the cell motility and dendricity phenotypes are interconnected, or reflect distinct signaling pathways. One possibility is that the enhanced motility of Tg+ melanophores results in the failure to properly remove focal adhesions that leads to the maintenance of processes and formation of dendritic morphology. Alternatively, the dendritic phenotype could result from de-differentiation of the melanophores to a more neural crest-like, progenitor state. Interestingly, our RNAseq data provides some support for both models. Specifically, it shows differential expression of both cell adhesion molecules and neuronal-associated genes between the Tg+ and Tg- melanophores. Obviously, additional analyses are required to explore these possibilities.

Notably, the most striking consequence of oncogenic GNAQ expression is the mislocalization of melanophores in the interstripe regions (Chapter 3) and also internally (Chapter 2). The appropriate localization of zebrafish melanophores is known to be controlled by a barrage of cues including repulsive, guidance, and apoptotic signals (Kelsh et al. 2009; Parichy and Spiewak 2015). When located in presumptive interstripe region, melanophores are subject to repulsive cues or, if they remain deeply embedded, apoptotic cues from xanthophore populations (Singh and Nusslein-Volhard 2015; Kelsh et al. 2009; Parichy and Turner 2003). The persistence of numerous Tg+ cells within the interstripe region of post-metamorphic fish indicates that these melanophores have acquired the ability to evade both signals. Notably, the
ability of \( Tg^+ \) melanophores to ignore the xanthophores’ repulsive cues is reminiscent of the phenotype of the \textit{obelix/jaguar} mutant, which affects the inwardly rectifying potassium channel 7.1, \textit{kir7.1} (Iwashita et al. 2006). It’s currently unclear how this mutation leads to stripe pattern disruption, but interesting by \textit{obelix/jaguar} has been placed downstream of \( \alpha_2 \)-adrenoceptor signaling (Iwashita et al. 2006), which creates obvious parallels to GNAQ signaling.

Our evidence for the increased survival of \( Tg^+ \) melanophores comes not only from the occupation of interstripes \textit{in vivo} but also from enhanced survival of purified cell populations \textit{in vitro}. This suggests a general, cell intrinsic shifting of the apoptotic threshold of these cells, rather than only a specific failure to detect the apoptotic signal from xanthophores. The effect of oncogenic GNAQ on cell survival has been previously studied and appears to be context dependent. While GNAQ expression in UM cell lines confers enhanced survival (Posch et al. 2015), the opposite is true in other cell types such as T cells (Wang et al. 2014) and, curiously, melanocytes in the interfollicular epidermis in mice (Huang, Urtatiz, and Van Raamsdonk 2015). This cell survival phenotype has obvious implications in cancer progression and further study of these observations could provide important insight into how oncogenic GNAQ/11 signaling mediates survival in the context of melanophores and UM.

Finally, our data suggest that the hyperpigmentation results, in large part, from an increase in total number of melanophores. The enhanced survival potential must certainly contribute to this increase, but our data suggest that this is also accompanied by an increase in production of these cells. This is most clearly established in the early embryos, where we see elevated melanophage numbers in the \( Tg^+ \) fish well before the developmental stage at which apoptosis of mislocalized melanophores is triggered, and is further illustrated by the progressive
increase in the melanophore representation in the flanks of metamorphic and adult fish.

Importantly, our gene expression analysis showed that genes associated with cell proliferation are upregulated in $Tg^+$ versus $Tg^-$ melanophores, providing a molecular mechanism for the observed melanocyte number increase. Taken together, it is easily conceivable how enhancement of the migratory, survival and proliferative properties of the melanophores by oncogenic GNAQ would facilitate transition towards tumorigenicity.
3.5. Methods

Zebrasfish lines

Experiments were performed in TAB5/14 (AB/Tübingen) zebrafish genetic background generated by Nancy Hopkins and Adam Amsterdam (Amsterdam et al. 1999). Tg(mitfa:GNAQ/11Q209L) zebrafish lines were generated and maintained as described previously with the exception that when tp53M214K mutation was not desired, lines were crossed back into the wild-type TAB5/14 genetic background (Chapter 2). Genotypes were determined by PCR after 8 weeks of age as described in Chapter 2. Experiments in this chapter were performed with fish from 2 lines out of the 20 transgenic lines recovered, designated Tg(mitfa:GNAQ209L-QE32A) and Tg(mitfa:GNA111Q209L-11U35).

Imaging zebrafish

For live and whole-mount imaging, fish were anesthetized and immobilized in methylcellulose as previously detailed (Chapter 2). For the image series throughout metamorphic development, individual fish were reared in plastic containers and fed rotifers twice daily. All fish were maintained at 28°C.

For the time-lapse movies of embryonic zebrafish, embryonic ~3dpf fish (approximately 78hrs post-fertilization) were mounted in 1.2% low melt agarose made in fish water to immobilize for time-lapse imaging. Images were taken at 4X every 20 minutes over the course of 14 hours in a temperature controlled chamber set to 28°C using a Nikon Eclipse TE3000 microscope with a SPOT-flex model 15.2 camera. Image and movie analysis was performed in FIJI.
**Background adaptation and analysis**

Embryonic zebrafish were raised in petri dishes maintained at 28°C under 14hrs on/10hrs off fluorescent light cycle conditions. Fish to be light- or dark-adapted were moved to petri dishes with white or black matte backgrounds along the underside and at least 1cm surrounding the dish. Background adaptation began at 4 dpf and fish were adapted for 24 hours prior to imaging at 5 dpf.

Images were taken on the backlit stereomicroscope as previously described under the same lighting and magnification settings for every fish in the experiment. Images were analyzed using threshold settings on FIJI image analysis software. Melanophore coverage and number were analyzed using the dorsal view of the fish in the area from the top of the eyes to the back of yolk sac (Supplemental Figure S3.11). This representative area was chosen for the fish as it was technically feasible to achieve acquisition of quality focused images of this region and was therefore the clearest for melanophore counting and analysis. Melanophore counting per zebrafish was only done in light-adapted conditions as this enabled easy distinction of single melanophores, while melanophore area was calculated in dark-adapted conditions in an attempt to use melanin dispersion as a proxy for cell morphology and spreading.

**Isolation and culture of melanophores from zebrafish fins**

Isolation and culture of melanophores from adult zebrafish fins was done almost entirely as published in Yamanaka, 2014. Briefly, tail, anal and pelvic fins were dissected from euthanized fish. The dissected fins were shaken with 1 mL trypsin solution [2.5 mg/mL trypsin (TRL; Worthington), 1.2 mg/mL BSA (Sigma), 1 mM EDTA in PBS] for 60 minutes at 28°C.
After 3 PBS washes, fins were vigorously shaken with collagenase solution [1 mg/mL collagenase I (CLS-1; Worthington), 0.1 mg/mL DNase I (DP; Worthington), 0.1 mg/mL soybean trypsin inhibitor (SI; Worthington), 1.2 mg/mL BSA in PBS] at 28°C for 60 minutes. The single-cell suspension was filtered using a 35-μm mesh strainer and centrifuged with 50% Percoll (Sigma) at 30 × g for 10 minutes to separate melanophores by density. The melanophore enriched pellet was resuspended in serum-free L15 (Gibco) medium and plated onto a collagen IV-coated 96-well dish (collagen from Corning catalogue #: 354233, rehydrated to make 100g/ml stock).

Time-lapse recording and analysis of melanophore migration and morphology in vitro

After 24 hours for seeding and spreading, the melanophore L15 medium was brought up to L15 medium with 10% FBS by exchanging one-half of the volume of the medium. Pigment cell movement was recorded on a NikonA1R confocal microscope using time-lapse microscopy every 20 minutes for 24 hours in a climate-controlled chamber at 29°C. Images were taken on an ANDOR black and white camera, to ensure that xanthophores were excluded from image analysis, every 4 hours an image detecting the presence of xanthophore autofluorescence (GFP) was taken. Images were recorded and integrated into stacks with NIS elements software and formatted for manual analysis of cell movement using the MTrackJ software on ImageJ. Cell movements were analyzed every hour.
**Image acquisition and analysis of in vitro cell viability**

For the purposes of quantitating cell viability in vitro, melanophores were isolated and plated on collagen-coated wells as described above. Cells were maintained at 28°C in 96-well dishes in L15. After allowing 24 hours for seeding and spreading, 4 images per well were taken daily at 10X magnification on a Nikon Eclipse TE2000-U microscope using a SPOT-RT3 camera model 25.2. Images were processed using SPOT analysis software. Live cells were counted in the 4 fields and averaged over the well as a proxy for cell number.

**Isolation of melanophores from zebrafish embryos for Coulter counter volume measurements and RNA purification**

In contrast to melanophores isolated from zebrafish fins with the intention of culturing, melanophore populations isolated for the purpose of volume measure by Coulter counter or RNA extraction for sequencing were purified from embryonic zebrafish populations. Between 400-1000 5 dpf embryonic fish were used per isolation depending on the number of embryos collected, the protocol was scaled appropriately to the number of fish. For melanophore purification of 1000 embryonic fish the protocol used was similar to Higdon et al., 2013 with some exceptions.

Anesthetized fish were washed in PBS and shaken for 20 minutes in 100 mL TrypLE Express (Invitrogen, 12604039) at 37°C. Eyes were separated from the larva by trituration and discarded to remove retinal pigmented epithelium contaminants. Remaining tissue was dissociated into single cell suspension as previously detailed, by vigorous shaking in collagenase solution at 28°C for 60 minutes. Dissociated cells were filtered through a 35μm screen and
pelleted by centrifugation in a swinging bucket rotor at 500 rcf for minutes at 4°C. Pellet was resuspended in 1 mL isotonic Percoll (Sigma) mixture prepared with 1 part 10X PBS to 9 parts Percoll. The resuspended pellet was transferred to Eppendorf tubes and centrifuged in a swinging bucket rotor at 2000rcf for 5 minutes at 4°C for density dependent separation. The pellet was resuspended in 400mL ice cold PBS and 2% fetal calf serum (FCS) and placed on top of a preformed Percoll density gradient. Preformed gradients were prepared by fixed-angle centrifugation in Eppendorf tubes at 10,000rcf for 15 minutes at 4°C. Eppendorf tubes with the cell suspension overlaid on top of the preformed Percoll gradient were centrifuged in a swinging bucket rotor at 2000rcf for 10 minutes at 4°C. After centrifugation, the Percoll should be aspirated leaving 100μL of volume over the melanophore pellet. Melanophores were brought up to 500μL volume in ice cold PBS with 2% FCS to wash, and kept on ice to be prepared for either the coulter counter or RNA purification. 10-20μL of melanophore suspension was sacrificed to be visualized on a hemocytometer to assess melanophore purity and yield. Typical rates of contamination by other cell types, typically xanthophores, ranged from 0-2%.

For volume dispersion quantitation by coulter counter, melanophores were isolated and diluted in Isoton II solution (Beckman) to give <12% saturation when processed with a Beckman Coulter Multisizer 3. About 16,000 cells per genotype were counted and sized. To decrease background signal and chances of including dead yeast or large debris, particles smaller than 8fL and larger than 20fL were excluded from data analysis. We used the mean and the standard deviation to plot the bar graph. This method of measuring cell volume was based on methods previously described in (Goranov et al. 2009).
To be prepared for RNA isolation, cells were spun down in swinging bucket rotor at 500 rcf for 5 minutes at 4°C. Residual Percoll and PBS wash was aspirated leaving 100μL of volume over the melanophore pellet, and then mixed with SuperaseIN RNA inhibitor (Invitrogen AM2694) to a final concentration of 20U/L in 250L of PBS with 2% FCS.

**RNA isolation, library preparation, Nextseq sequencing and data processing**

RNA was isolated following the protocol for the Qiagen RNeasy Micro kit. Samples were quantified on advanced analytical fragment analyzer to verify quality and concentration prior to proceeding with library preparation. RNAseq libraries were prepared as detailed in Chapter 2.

For analysis following the sequencing, RNAseq data were aligned and summarized using bowtie2 version 2.2.3 (Langmead and Salzberg, 2011), RSEM version 1.2.25 (http://deweylab.github.io/RSEM/) and an Ensembl annotation file for the Zf10 genome assembly. Differential expression analysis was done with R version 3.2.2 and DESeq_1.22.0 (Anders and Huber, 2010). Overrepresented process networks (https://portal.genego.com/) or GO categories (https://portal.genego.com/, https://david.ncifcrf.gov/) were identified using the indicated online tools and a reference set consisting of human orthologs examined in the analysis. GSEA was run using the desktop Java implementation version 2.2.1. Expression data were row-centered using the Preprocess Dataset Module of the Broad Institute’s public GenePattern server. Clustering (Ward’s Method) and other data presentation was done using TIBCO Spotfire version 6.5.3.
3.5.1. Acknowledgements

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This work was supported by the Melanoma Research Alliance. Dahlia Perez was supported by an NSF fellowship and a D.H. Koch Graduate Research Fellowship.
Supplementary Movies S3.1 and S3.2. Melanophore motility across the yolk sac of day 3.5-4 dpf embryonic zebrafish. Please refer to supplemented drive to view time-lapse imaging of embryonic zebrafish from approximately 78 hours post fertilization to 92 hours post fertilization. Melanophore migrate across the yolk sac down towards the ventral area of the fish in $Tg^-$ zebrafish and migrate more slowly or not at all in $Tg^+$ fish.
Supplementary Figure S3.3. \textit{Tg(mitfa:GNA11^{Q209L})} zebrafish display pigmentation defects throughout development. (A) Embryonic zebrafish in an \textit{II}^{Q209L} transgenic line display hyperpigmentation in the dorsal view of the fish (5 dpf). This phenotype also begins at 3 dpf (data not shown). (B) Metamorphic \textit{II}^{Q209L} zebrafish are hyperpigmented in both the dorsal and lateral view. Stripe boundary formation is also disrupted as melanophores localize the interstripe region compared to \textit{Tg} clutchmate controls (28 dpf). (C) The \textit{II}^{Q209L} juvenile adult has non linear stripes and many more melanophores, particularly in the dorsal flank area, compare to \textit{Tg} clutchmate controls (6 weeks post fertilization).
Supplementary Figure S3.4. *Tg(mita:GNAQQ209L)* zebrafish respond to exogenously applied epinephrine. (A) Images of *Tg* and *Tg*+ embryonic (5 dpf) zebrafish exposed to 10mM epinephrine. (B) When exposed to 10mM epinephrine, *Tg* zebrafish respond by quickly contracting melanin within melanophores, followed by a brief relaxation, and then prolonged contraction. *Tg*+ melanophores respond in this same manner but with delayed kinetics and a greater amplitude of response.
Supplementary Figure S3.5. Background adaptation assays in \textit{tp53^{M214K/M214K}} mutant background reveals that \textit{tp53} mutation has no effect on melanin coverage and melanophore number in \textit{Tg(mita:GNAQ^{Q209L})} zebrafish. For brevity, \textit{tp53^{M214K/M214K}} labeled \textit{tp53}^{\mathrm{--}} in all figures. A) \textit{Tg};\textit{tp53}^{\mathrm{--}} and \textit{Tg};\textit{tp53}^{\mathrm{+/--}} zebrafish were background adapted from 4-5 dpf. (B) \textit{Tg};\textit{tp53}^{\mathrm{--}} and \textit{Tg};\textit{tp53}^{\mathrm{+/--}} embryonic fish have no difference in melanin coverage when compared to \textit{Tg} and \textit{Tg}^{+} controls respectively. Regardless of background, all genotypes expand and contract melanin properly over dark/light conditions. (C) \textit{Tg};\textit{tp53}^{\mathrm{--}} and \textit{Tg};\textit{tp53}^{\mathrm{+/--}} embryonic fish have no difference in area of melanin coverage per melanophore when compared to \textit{Tg} and \textit{Tg}^{+} controls respectively. (D) \textit{Tg};\textit{tp53}^{\mathrm{--}} and \textit{Tg};\textit{tp53}^{\mathrm{+/--}} embryonic fish have no difference melanophore number per fish when compared to \textit{Tg} and \textit{Tg}^{+} controls respectively.
Supplementary Figure S3.6. Tg(mifA:GNA4Q209L) zebrafish display hyperpigmentation, failure to establish linear stripes, and melanophore mislocalization phenotypes in the fins. In non-transgenic zebrafish, cutaneous stripes extend back into the tail fin in the same stripe-interstripe-stripe pattern. In contrast, Tg+ zebrafish fins appear disordered, hyperpigmented and some melanophores fail to localize away from the repulsive xanthophores. These phenotypes mimic those observed in the skin. Scale bar = 500μm.
Supplementary Figure S3.7. Non-transgenic melanophores expand and contract melanin within the cell boundaries. (A) Time-lapse images of 2 Tg melanophores imaged every hour reveal changes in melanin dispersion over time. At timepoints 0:00, 3:00, 4:00 and 5:00 the top melanophore appears to have the melanin drawn into the cell into a punctate area. In contrast at time points 1:00, 2:00, 6:00-9:00 the melanin is more dispersed. To know accurate if the melanized area is accurately reflecting the cell boundaries staining for cytosolic or cell boundary marker must be done. (B) Staining of phalloidin in Tg melanophores reveals that often the cell boundary extends beyond the area of melanin dispersion. Scale bar = 100μm.
Supplementary Movies S3.8 and S3.9. Time-lapse imaging of non-transgenic and
\( Tg(\text{mitfa:GNAQ}^{Q209L}) \) expressing melanophore motility \textit{in vitro}. Please refer to supplemented
drive to view time-lapse imaging of \( Tg^- \) and \( Tg^+ \) melanophore motility \textit{in vitro}. (3.8) \( Tg^- \)
melanophores exhibit a modest capacity for motility. Many cells also expand and contract their
melanin within their borders. (3.9) \( Tg^+ \) melanophores are extremely motile. Melanophores can
also undergo stunning changing in melanophore morphology. Time-lapse images were taken at
10X magnification every 20 minutes for 36 hours.
Supplementary Figure S3.10. Conversion from zebrafish to human gene symbols. (A) Graph and table both representing the number of zebrafish genes in the dataset for which it was necessary to chose one ortholog for functional analysis. We had a list of 11,373 1:1 human to fish matched gene symbols to move forward with after this selection. (B) Graphical representation of how orthologs were selected. (b') The ortholog in pink is differentially expressed and therefore selected (b'') neither ortholog is differentially expressed, therefore the one with the highest gene expression is selected (pink).
Supplementary Figure S3.11. Functional analysis using Gene-go and DAVID of differentially expressed genes between transgenic and nontransgenic melanophore populations. (A) Top 5 networks revealed by Gene-go analysis of differentially expressed genes between Tg^<sup>+</sup> and Tg^- melanophore populations. (B) Top 10 GO categories revealed by DAVID analysis of differentially expressed genes plotted against adjusted p-value (Benjamini). See methods section for details on functional analysis.
Supplementary Figure S3.12. Schematic and images to demonstrate calculation of melanophore coverage per fish for background adaptation assays. Zebrafish were imaged using a backlit stereomicroscope set to the same zoom and lighting conditions throughout the experiment. Images were analyzed using the thresholding and measuring capabilities of FIJI. (a’) The representative area of the fish for the calculations includes the head and extends back towards to last visible region of the yolk sac as judged from the dorsal view. (a’”) After this area was quantitated, the melanophores in this area, up to the region behind the eyes, where encircled and the thresholding tool was used to select and measure pixels with intensity values that fall within the range of intensity consistent with pigmentation. This range is kept constant throughout analysis of the experiment. The area of melanophore coverage is then normalized to the total area of the fish to give the final measurement of % melanophore coverage.
3.7. References


Chapter Four

Conclusions and Future Directions
Chapter 4: Table of Contents

4.1. Overview..................................................................................138

4.2. Ongoing and future experiments to interrogate the role of candidate genes of interest from RNAseq analysis in UM tumor progression.........................141

4.2.1. Candidate genes of particular interest ........................................142

4.2.2. Ongoing and future experiments ..................................................147

4.3. Conclusions and potential experiments utilizing phenotypic changes in transgene expressing zebrafish.......................................................149

4.3.1. Future experiments using zebrafish melanophore biology to elucidate the role of GNAQ\textsuperscript{209L} expression in signaling, proliferation and cell size..149

4.3.2. Chemical screens utilizing Tg(mitfa:GNAQ/11\textsuperscript{209L}) embryonic phenotypes .................................................................152

4.4. Ongoing efforts to improve our zebrafish model of UM.....................152

4.5. References...................................................................................155
4.1. Overview

Within the last 10 years important advances have been made in understanding the biology of uveal melanoma (UM). In particular, the identification of oncogenic mutations in $GNAQ$ and $GNA11$ as the most commonly occurring driver mutations (Van Raamsdonk et al. 2009; Van Raamsdonk et al. 2010) has allowed development of better tools to analyze this disease. However these leaps in understanding have yet to be translated into the discovery of new treatments that show promise in clinical trials. Therefore, the continuation of research and the diversification of methodologies used in this field is paramount in defining candidate treatments that could be tested in the clinic. To this end, in this thesis I describe our work to generate a zebrafish model of UM and the use of this model to expand our understanding of UM and the oncogenic role of $GNAQ/11$ mutations.

Prior to the identification of oncogenic mutations in $GNAQ$ or $GNA11$ models of UM available for research were restricted to human patient-derived cell lines studied both in an in vitro context and in vivo orthotopic injection (Luyten et al. 1996; Yang et al. 2008); and only in the past year has the first vertebrate model of UM been published in mice (Huang, Urtatiz, and Van Raamsdonk 2015; Feng et al. 2014). However, using the zebrafish as a cancer model offers many advantages over mouse models that can be utilized to address the molecular mechanisms in cancer in complementary or orthogonal work. Therefore, we created a zebrafish model of UM to generate a novel tool for UM research. Our zebrafish model of UM is driven by melanocyte-specific expression of two of the oncogenic point mutants found in human patients, namely $GNAQ^{Q209L}$ or $GNA11^{Q209L}$. We generated this model using Tol2-mediated transgenesis to
mediate insertion of a transgene containing the melanocyte specific \textit{mitfa} promoter upstream of the indicated oncogene into the zebrafish germline, and subsequently crossed these transgenic fish, \textit{Tg(mitfa:GNAQ/Q209L)} indicated as \textit{Tg+}, into the \textit{tp53}^{M214K/M214K} mutant background in order to facilitate tumorigenesis (Chapter 2). We demonstrate that \textit{Tg(mitfa:GNAQ/Q209L);tp53}^{M214K/M214K} fish develop uveal melanomas with similar morphology to human tumors, as well as a wide variety of other aggressive, pigmented tumors. These other tumors vary in location and appear in many organs and tissues of the zebrafish including the skin, liver, pancreas, kidney, and skeletal muscle. Regardless of location, these tumors all express high levels of \textit{GNAQ} or \textit{GNA11} as validated by IHC and RNAseq and also overexpress nuclear \textit{YAP}, an established downstream target of oncogenic \textit{GNAQ}/\textit{GNA11} signaling thus recapitulating the human malignancy. Therefore, we report the successful generation of a zebrafish model of UM.

Using this zebrafish model of UM, I embarked on experiments to further our understanding in how oncogenic \textit{GNAQ}/\textit{GNA11} expression alters melanophore biology. For these experiments, I used \textit{Tg(mitfa:GNAQ/Q209L)} zebrafish in the absence of \textit{tp53} mutation to try to specifically address how the \textit{GNAQ/Q209L} oncogene significantly alters the fundamental biology of melanophores in ways that could facilitate tumor initiation. I observed that upon melanophore-specific expression of either \textit{GNAQ/Q209L} or \textit{GNA11/Q209L}, zebrafish developed a striking phenotypic defect in pigmentation as early as 3 dpf that persisted, and grew more dramatic, into adulthood (Chapter 3). These gross phenotypic changes in pigmentation throughout development are consistently the result of hyperpigmentation, mislocalization of melanophores and a failure to respect boundary cues. Taking advantage of the innate ability of zebrafish to expand or contract their melanosomes, I succeeded in quantifying melanosome
expansion and melanophore number in melanin expanded and contracted states respectively. I discovered that hyperpigmentation in Tg\(^+\) animals is due both to an increase in melanophore number and an increase in melanin distribution within cells. Additionally, I observed that melanophores in Tg\(^+\) embryos fail to migrate from the yolk sac between 3.5 and 4.5 dpf and that melanophores are located inappropriately in the interstripe region during the metamorphic stage and in the adult. During metamorphosis, the embryonic melanophores of zebrafish undergo apoptosis or migrate as part of the formation of the adult stripe pattern. Tg\(^+\) melanophores survived in the zebrafish interstripe while Tg\(^-\) melanophores cleared the interstripe appropriately, through migration or apoptosis which is known to occur. Together these results indicate defects in proliferation and cell signaling both of which are significant as the pathways responsible for these phenotypes are frequently deregulated in human cancers.

To further investigate these phenotypes, I generated an in vitro system in which to interrogate oncogenic GNAQ/11 function by adapting previous methods to isolate populations of melanophores for cell culture (Yamanaka and Kondo 2014; Higdon, Mitra, and Johnson 2013). Importantly, these isolated melanophores confer a significant advantage over previously derived tumor cell lines because they neither have, nor require, additional modifications or mutations for culture. Therefore, the effects of oncogenic GNAQ/11 mutations can be assessed without the concern that exogenous transforming factors influence the analyses. I determined that Tg\(^+\) melanophores do not have a greater volume than Tg\(^-\) melanophores even though they do have significantly increased area of pigment dispersal in 2D culture. These results indicate that the hyperpigmentation phenotype is not likely due to changes in cell volume. Additionally, I observed in vitro that Tg\(^+\) melanophores display a more dendritic morphology, increased motility...
and increased survival compared to Tg\(^+\) controls. Taken together, these results implicate oncogenic GNAQ/11 signaling as an effector of tumorigenic pathways such as cell signaling, cell motility, and cell viability.

Having utilized our zebrafish model of UM to uncover a diversity of potentially tumorigenic phenotypes downstream of GNAQ/11\(^{Q209L}\) mutation, we were interested to understand the mechanisms by which oncogenic GNAQ/11 effects these changes. To this end, I generated populations of purified Tg\(^-\) and Tg\(^+\) melanophores for transcriptome sequencing and subsequent differential gene expression analysis. Our results show significant changes in the expression of 353 genes between Tg\(^-\) and Tg\(^+\) melanophores, and gene set enrichment analysis implicates GNAQ\(^{Q209L}\) expression in differentiation, proliferation, cell adhesion and signal transduction. In the next section, I will discuss ongoing work to assess the roles of genes and pathways of interest implicated by the RNAseq analysis using in vitro and in vivo assays.

### 4.2. Ongoing and future experiments to interrogate the role of candidate genes of interest from RNAseq analysis in UM tumor progression

In Chapter 3, I described the successful generation and analysis of RNAseq data from purified Tg\(^-\) and Tg\(^+\) melanophore populations. It is well known that G-protein coupled receptors (GPCRs) and their downstream effectors, such as GNAQ/11, mediate a diverse set of downstream signaling changes and cellular processes (Rozengurt 2007; Katritch, Cherezov, and Stevens 2013; Hanlon and Andrew 2015). Therefore, it comes as no surprise that research into the effects of oncogenic GNAQ/11\(^{Q209L}\) signaling reveals pleiotropic changes in melanophore biology. As previously discussed, the diversity of these phenotypic changes is reflected in the
diversity of the differentially expressed genes (DEGs) and GSEA categories identified by comparing transgenic versus non-transgenic melanophore populations.

In the following section I will discuss which candidate DEGs from this RNAseq data set are of special interest and conclude by discussing ongoing and future experiments designed to enhance our understanding of how expression of GNAQ/11Q209L promotes tumorigenesis.

4.2.1. Candidate genes of particular interest

Granulin (GRN):

Granulin (GRN) belongs to a family of secreted, glycosylated peptides that are cleaved from a single precursor protein, progranulin (Bateman and Bennett 2009). This gene stood out due to the finding that of the four human-GRN homologs in the zebrafish, three are significantly up-regulated in Tg+ melanophores relative to Tg- melanophores. Of the three up-regulated GRNs in zebrafish, two of them, GRN2 and GRNJ, are the number two and number three ranked genes with the largest log2 fold change upon GNAQQ209L expression (Table 3.1). This consistent and dramatic change in GRN expression suggest that the GRN family is coordinately up-regulated by GNAQQ209L expression.

Interestingly, GRN is frequently expressed under conditions of tissue remodeling in which cells are actively dividing and migrating (Bateman and Bennett 2009). Notably, high GRN levels are present in the adult epithelia in regions that are rapidly turning over, such as epidermal keratinocytes and intestinal crypts (Daniel et al. 2000). In the same vein, GRN expression is typically very low in quiescent endothelial cells, however, upon wounding these cells initiate a rapid remodeling, proliferative and migratory response during which time GRN is dramatically
up-regulated (He et al. 2003). In addition to these tissues, GRN is highly expressed in non-proliferative neuronal tissues, indicating that GRN likely plays a variety of context-dependent roles to mediate different cellular processes in different tissues. Though the relationship between GRN and neurodegenerative disease is not entirely understood, it is generally believed that GRN plays a role in neuronal survival as GRN depletion in neurons results in atrophy of the frontal and temporal lobes (Eriksen and Mackenzie 2008). The reported biological activities of GRN fall into three main categories, namely: growth factor-like activities, immune responses and neuronal effects (Cenik et al. 2012). Considering the effects of GRN expression in stimulating proliferation, motility and wound healing, it comes as no surprise that GRN is overexpressed in many cancers including carcinomas, gliomas and sarcomas (Cenik et al. 2012).

GRN-mediated promotion of cell proliferation includes activation of ERK, P13K and Akt signaling pathways in neurons as well as in tumor cells (Bateman and Bennett 2009; Cenik et al. 2012). Encouragingly, these pathways are also known to be downstream of GNAQ/11 signaling (Saraiva et al. 2005; Shoushtari and Carvajal 2014; Van Raamsdonk et al. 2009). Through careful phenotypic analysis and quantitation, I demonstrated in vivo that zebrafish expressing GNAQ/11Q209L have more melanophores than their non-transgenic clutchmate controls. This result implicates oncogenic GNAQ/11 signaling in promoting cell proliferation. GSEA analysis of RNAseq from Tg+ and Tg- melanophore populations further substantiates this result, as the most significant GSEA categories identified are those associated with cell cycle regulation. Currently we do not know which DEGs, if any, are responsible for an increase in cell proliferation downstream of GNAQ Q209L expression. GRN represents an excellent candidate for further study. While I have highlighted GRN as a potential mediator of cell-proliferation, clearly
the possibility exists that GRN could also be an effector of the cell motility and/or cell survival phenotypes exhibited by \textit{Tg}^+ melanophores (Chapter 3).

To interrogate the potential role of GRN in our model of UM several approaches are conceivable. Unfortunately, it is not possible to study the proliferative effects of GRN \textit{in vitro} with my current model because these primary melanophores isolated from fish do not proliferate in culture. It is also worth noting that generally melanophores do not proliferate in the skin once they are fully differentiated. However, there exists a niche of proliferative melanophore stem cells (MSC) associated with peripheral nerves identifiable by \textit{mitfa} reporters or the \textit{mitfa} transcript (Budi, Patterson, and Parichy 2011). To address the hypothesis that GRN up-regulation affects proliferation we first would assess GRN expression levels, \textit{GNAQ/11Q209L} transgene levels, and markers of proliferation in that MSC population by IF or IHC in tissue sections. Interrogation of the potential effects of GRN on cell motility can be pursued \textit{in vitro}. As GRN is a secreted protein, it is formally possible that GRN acts upstream of \textit{GNAQ/11} activation. To test this possibility, we could assess the consequences of exogenously applied GRN protein upon motility in \textit{Tg}^+ or \textit{Tg}^- melanophores isolated from fish.

Finally, though GRN overexpression has been implicated in many cancers, it has not yet been implicated in UM. To assess the relevance of GRN overexpression in the context of UM tumors, I propose to first examine the expression of GRN protein in melanomas from \textit{Tg(mitfa:GNAQ/11Q209L);tp53M214K/M214K} zebrafish by IHC. Follow-up experiments may involve expressing shRNAs or using CRISPR/Cas9 technology to test if loss of \textit{GRNs} impacts melanophore biology or tumor formation. In addition to utilizing our zebrafish model of UM,
analyses of human profiles of UM tumors in The Cancer Genome Atlas (TCGA) will be conducted when these data become publicly available.

**Neuronal Calcium Binding Protein-1 (NECAB1):**

As the name would suggest, Neuronal Calcium Binding Protein 1 (NECAB1) plays a role in Ca\(^{2+}\)-binding in neuronal cells. NECAB1 belongs to a subfamily of neuronal proteins with one N-terminal EF-hand motif, a helix-loop-helix motif commonly found in calcium-binding proteins, and a C-terminal monooxygenase domain (Sugita, Ho, and Sudhof 2002). Unlike other members of the family, NECAB1 is localized almost exclusively to neuronal tissue (Sugita, Ho, and Sudhof 2002). More recent studies report NECAB1 expression in excitatory neurons, motor neurons and neurons of the dorsal root ganglia among other classes (Zhang et al. 2014; Zhang et al. 2016). Though the function of NECAB1 is not well known, data mining in the Human Protein Atlas (HPA) and TCGA reveals that NECAB1 is moderately up-regulated in gliomas.

NECAB1 represents the 10th most up-regulated gene upon Tg\(^{+}\) expression in melanophores by Log2 fold change (Chapter 3, Table 3.3.1). As previously mentioned, melanophores are neural crest-derived cells and therefore share a common progenitor with neurons (Singh and Nusslein-Volhard 2015). Class II UM, those that exhibit poor prognosis, appear to have a more de-differentiated neuronal phenotype (Harbour et al. 2010). RNAseq analysis of Tg\(^{+}\) and Tg\(^{-}\) melanophore populations revealed expression changes in many neuronal genes, including NECAB1. We would be very interested to test if oncogenic GNAQ/11 mutation leads to an increase in expression of neuronal genes, as the RNAseq suggests, and if this is part of a de-differentiation program in the Tg\(^{+}\) expressing melanophores. One approach to test the
role of NECAB1 expression in $Tg^+$ melanophores in vivo would be to inject $Tg^+$ embryonic fish with a mitfa promoter based plasmid driving shRNA expression to knockdown NECAB1 levels. In this manner, I could visualize the effects of NECAB1 melanophore specific knockdown in the $Tg^+$ background and understand the contribution of NECAB1 overexpression to the embryonic $Tg^+$ melanophore phenotypes. A phenotypic reversion in cell number, motility or morphology in these zebrafish would implicate NECAB1 in these potentially tumorigenic cell processes. Furthermore, as with GRN, I would assess NECAB1 expression in the zebrafish melanomas, and interrogate publicly available data to determine the relevance to the human disease.

**Adhesion G-Protein Coupled Receptor G7 (ADGRG7):**

Adhesion G-Protein Coupled Receptor (ADGRG7) is the most down-regulated gene in $Tg^+$ melanophores relative to $Tg$ melanophores according to the RNAseq data (Chapter 3, Table 3.3.2). Also called GPR128, ADGRG7 is a orphan adhesion-GPCR (Hamann et al. 2015). Adhesion GPCRs (aGPCR) represents one of the five families of GPCRs, and are characterized by noncanonical or unusual protein structure (Langenhan, Aust, and Hamann 2016). The 33 known aGPCRs are further classified into nine families according to the molecular signature of their 7-transmembrane domain and extracellular domains. Work utilizing genetic models in zebrafish, mouse and *C. elegans* work have begun to establish a role for aGPCRs in cell adhesion and migration (Langenhan, Aust, and Hamann 2016; Carreira-Barbosa et al. 2009). Generally, aGPCRs have fairly ubiquitous expression, indeed human ADGRG7 exhibits expression throughout many tissues according to the Human Protein Atlas. ADGRG7 expression has been characterized in zebrafish as part of an effort to ascertain the spatio-temporal location of all
aGPCRs in zebrafish and appears to be expressed in the kidney and in the skin (Harty et al. 2015). To follow up on this observation I would first assess whether or not ADGRG7 is expressed in melanophores or in other cell-types within the zebrafish skin.

It is tempting to hypothesize that ADGRG7 could be upstream of GNAQ/11 signaling and is being down-regulated by the cell as part of a feedback mechanism in an attempt to turn off oncogenic GNAQ^{Q209L} or GNA11^{Q209L} signaling. However, formally, GPCR signaling through the G trimer has only been formally demonstrated for a handful of aGPCRs (Langenhan, Aust, and Hamann 2016). Nevertheless, using a genetic approach to up-regulate ADGRG7 in vivo in melanophores of Tg^{+} embryonic zebrafish would provide information as to whether or not downregulation contributes to their phenotype. If GNAQ/11^{Q209L}-mediated signaling has not exhausted the cell, perhaps signaling though forced ADGRG7 expression would activate endogenous GNAQ/11 and exacerbate the Tg^{+} melanophore phenotypes. Alternatively, ADGRG7 expression may antagonize GNAQ/11^{Q209L}-mediated proliferation, cell adhesion or migration phenotypes.

### 4.2.2. Ongoing and future experiments

In addition to interrogating the aforementioned genes of interest, in this section I will discuss ongoing and future efforts to exploit the RNAseq data that we have already generated in Tg^{−} and Tg^{+} melanophore populations to elucidate the role of oncogenic GNAQ/11 mutation as an initiating event. Zebrafish provide a powerful genetic tool in which to analyze DEGs of interest following analysis of these RNA data. One intriguing possibility would be to use a miniCOOPR-type assay to perform a small genetic screen to assess the impact of genes of
interest on tumorigenesis (Iyengar, Houvras, and Ceol 2012; Ceol et al. 2011)(Chapter 1). This approach would generally involve using Tol2-mediated transgenesis to integrate the miniCOOPR plasmid containing a gene of interest and the mitfa ORF behind the mitfa promoter into the genome of a nacre^−/−, Tg(mitfa:GNAQ/11Q209L), tp53^M214K/M214K mutant zebrafish. Though this would be the version of this experiment truest to original protocol, I believe various iterations of this kind of experiment would be informative. For example, as alluded to in the discussion of ADGRG7, I believe it would be interesting to perform these experiments in embryonic fish in a nacre^−/−, Tg(mitfa:GNAQ/11Q209L) background in the absence of tp53 mutation. Based on my previous findings, this experiment could use readouts such as melanophore number, morphology and motility to inform us how the gene of interest cooperates or antagonizes with GNAQ/11Q209L expression. Another advantage of these types of experiments in embryonic fish is their quick turnaround, an experiment such as this could be completed by 5 dpf.

Lastly, while transcriptome analysis is a tried and true tool of current biologists, not all intracellular changes are reflected by changes in gene expression. While many signaling cascades can result in a change in transcriptional programs as their end effect, undoubtedly there are signaling changes downstream of oncogenic GNAQ/11 signaling that require a different means of interrogation to bring them to light. Common proteomic approaches including analyzing proteome-wide phosphorylation events would be ideal to study the effects of oncogenic GNAQ/11 signaling, however, it is worth noting the technical challenges of this type of experiment. The number of melanophores collected from a single purification from 400-1000 embryonic fish ranges from 50,000 to 140,000 cells, and while this is more than enough material for RNAseq using available technology this small amount of cellular material could pose as an obstacle to
phospho-mass spectrometry experiments. Regardless, the RNAseq data set has already allowed the interrogation of oncogenic GNAQ/11 signaling.

4.3. Conclusions and potential experiments utilizing phenotypic changes in transgene expressing zebrafish

In Chapter 3, I detail our efforts to learn about alterations in melanophore biology resulting from oncogenic GNAQ/11 signaling. Both in vivo and in vitro experiments implicate GNAQ/11Q209L expression in alterations in cell signaling, cell proliferation and cell motility and morphology. In this next section, I will discuss future experiments that would expand the work I have presented thus far. I will focus my discussion around experiments that will continue to utilize the zebrafish stripe patterning phenotypes to elucidate the effect of oncogenic GNAQ/11 expression.

4.3.1. Future experiments using zebrafish melanophore biology to elucidate the role of GNAQQ209L expression in signaling, proliferation and size

As previously demonstrated in Chapter 3, in vitro time-lapse analysis reveals a striking increase in motility in Tg+ melanophores over Tg- controls. While this effect is clear, the mechanisms underlying it are not. Genes that could potentially address this phenotype are present in the RNAseq data set. Examples of such genes of interest include GRN (granulin) as discussed in the previous section. However, there are aspects of the zebrafish phenotype that are not explained by this change in motility. For example, the delay in off melanophore migration off the yolk sac between 3.5- 4.5 dpf in Tg+ embryonic zebrafish seems to be in opposition to the clear ability of Tg+ melanophores to migrate more in vitro. Additionally, the presence of melanophores in the interstripe of the adult and metamorphic pattern also seems to contradict the
enhanced ability of \( Tg^+ \) melanophore to move. This last \textit{in vivo} observation suggests that, besides an increased capacity for cell survival despite apoptotic cues, the \( Tg^+ \) melanophores also fail to respond properly to repulsive cues from neighboring xanthophores.

The \textit{in vitro} work clearly shows that \( Tg^+ \) melanophores are capable of migration, therefore further experimentation taking into consideration the melanophore-xanthophore cell-cell signaling involved in melanophore migration in the \textit{in vivo} context could help reconcile these results. To test the hypothesis that \( Tg^+ \) expressing melanophores, while capable of motility, are unresponsive to xanthophore-derived repulsive cues, I would collect time-lapse images of a \( Tg^- \)-xanthophore-\( Tg^+ \) melanophore pairs \textit{in vitro} as Yamanaka and Kondo describe in their 2014 paper (Yamanaka and Kondo 2014). Cell movement recordings of wild-type xanthophore-melanophore pairs reveals a run-chase interaction in which melanophores move away from xanthophores that extend a pseudopod to touch the melanophore and deliver a repulsive cue. However, melanophores from the \textit{jaguar} mutant, also characterized by xanthophores and melanophores mingling in the interstripe border regions, move around xanthophores rather than away from them (Yamanaka and Kondo 2014). This experiment would test if GNAQ/11 signaling functions in directed cell movement in addition to motility, thereby bridging a role for GNAQ/11\textsuperscript{Q209L} in both cell motility and intracellular signaling.

In Chapter 3 I described evidence of GNAQ/11\textsuperscript{Q209L} signaling in cell proliferation \textit{in vivo}. As previously mentioned, in this model system it is not possible to study cell proliferation \textit{in vitro} as neither \( Tg^- \) nor \( Tg^+ \) melanophores ever divide in culture. Taken together, the \textit{in vivo} data, including the increased melanophore number in embryonic fish, combined with the GSEA analysis implicating the deregulation of cell cycle regulation pathways are compelling. However,
to directly probe cell proliferation, we could stain sections from metamorphic fish with markers of cell proliferation and quantification, as described in published studies (Budi, Patterson, and Parichy 2011). An increase in proliferative markers in mitfa+ groups of cells by the dorsal root ganglia in Tg+ animals would validate the hypothesis that GNAQ/11Q209L signaling enhances proliferation in our model.

Lastly, we have shown that while Tg+ melanophores have a larger area of pigment distribution both in vivo and in vitro, the cells themselves are not bigger than Tg- melanophores when measuring size by volume displacement. While this trend in melanin dispersal and melanophore spreading in Tg+ is clear, the underlying cause is as yet unknown. The first possible explanation could be an increase in melanin synthesis, however there is no evidence that an increase in melanin production is downstream of oncogenic GNAQ/11Q209L signaling in this model from the RNAseq analysis. Of course that does not entirely preclude the possibility that changes in melanin synthesis are translational rather than transcriptional, but the RNAseq data do make this explanation less likely. An alternative hypothesis, based on the RNAseq data, could be that Tg+ expression misregulates cell-matrix adhesion thereby affecting cells spreading. Indeed, ‘cell adhesion integrin-mediated cell-matrix adhesion’ and ‘KEGG-cell adhesion molecules’ are examples of two enriched gene sets as revealed by Gene-go and GSEA analysis respectively. Further work to interrogate the genes of interest in these categories, possibly by in vivo screening as previously suggested, is necessary to connect this strong trend in the RNAseq analysis with our phenotypic data.
4.3.2. Chemical screens utilizing Tg(mitfa:GNAQ/11Q209L) embryonic phenotypes

The zebrafish embryo is an ideal system in which to perform chemical screens. Among other advantages, zebrafish embryos are easily attainable, develop externally from the mother, and phenotypes can be easily screened using brightfield, *in situ* hybridization or fluorescence assays. Through thorough characterization of our model zebrafish model of UM we have shown that fish expressing Tg(mitfa:GNAQ/11Q209L) have a variety of strong early embryonic phenotypes; therefore, we believe that this model of UM could be a potentially powerful platform in which to perform a screen. I have previously discussed the possibility of small genetic screens using miniCOOPR-like strategies, however the phenotypes presented in Chapter 3 may be amenable to a chemical screen as well. Chemical screens of this nature have been performed before; for example, White and colleagues successfully utilized their model of BRAF driven melanomas to screen a library of about 2,000 for inhibitors of neural crest programs by *in situ* hybridization (White et al. 2011). For our model of UM, a reasonable output for a screen of this kind would be either the melanophore phenotype itself, or *in situ* hybridization of a target gene thought to act as proxy for UM tumorigenesis determined based on proposed RNAseq analysis of data from tumors of Tg(mitfa:GNAQ/11Q209L);tp53M214K/M214K zebrafish.

4.4. Ongoing efforts to improve our zebrafish model of UM

To further our understanding of the role of GNAQ/11Q209L mutation in the context of tumor progression, we are interested in expanding the transcriptome analysis to include melanophore populations in the presence of tp53M214K/M214K mutation and, importantly, to include the tumors themselves. Indeed, this is already underway; however, our initial analysis of
RNAseq data from melanomas in our zebrafish UM model indicated that analyzing more tumors would increase our statistical power in this heterogeneous tumor population. Once we have collected RNAseq data from the tumors, we will be very interested to compare expressed genes in all of our Tg melanophore, Tg melanophore, Tg;tp53M214K/M214K melanophore, and tumor samples to elucidate the gene expression changes through different phases of tumorigenesis. Furthermore, it would also be interesting to compare our data with the phenotypes and transcriptome from Tg(mitfa:BRAFV600E) melanophores as well as Tg(mitfa:BRAFV600E); tp53M214K/M214K melanomas given that common tumor type and selective pressure for GNAQ/11 mutations in UM versus BRAF mutations in epidermal melanomas.

Another important question in the study of UM in addition to the role of GNAQ/11 is how cooperating mutations contribute to tumorigenesis. The importance of this question is underscored by the fact that while the primary tumor is often treatable, metastatic UM is, thus far, untreatable. As discussed in the introduction, significant progress in uncovering cooperating mutations in UM has been made, however, their roles in UM and how they could be utilized therapeutically are not yet fully understood. Mutation of BAP1 stands out among the cooperating mutations as an excellent predictor of Class II UMs and poor prognosis (Harbour et al. 2010). To investigate the role of BAP1 in our model we have generated a germline mutation in BAP1 using CRISPR/Cas9 technology and have crossed it into our Tg(mitfa:GNAQ/11Q209L); tp53M214K/M214K model. While it is too early to discuss the effects of BAP1 mutation on our zebrafish model of UM, we are optimistic that this tool will assist us in better recapitulating the human metastatic disease and in furthering our investigation of cooperating mutations in UM.
Thus, in this thesis I have described analyses of a novel animal model of UM driven by expression of oncogenic forms of \textit{GNAQ} and \textit{GNA11}. However, we are only beginning to use this model to its full potential. The acquisition of this model has led to more intriguing questions, including; how the genes of interest generated from RNAseq analysis are involved in UM tumorigenesis, how to continue to probe and utilize the strong phenotypic characteristics of oncogenic GNAQ characteristics in future screens and how the UM model can be expanded to elucidate the role of cooperating mutations in UM.
4.5. References


Yamanaka, Hiroaki, and Shigeru Kondo. 2014. 'In vitro analysis suggests that difference in cell movement during direct interaction can generate various pigment patterns in vivo', *PNAS*, 111: 1867-72.

