10-6-2015

Insights into Melanocyte Regeneration and Melanoma Initiation Using the Zebrafish Model System: A Dissertation

Sharanya Iyengar

University of Massachusetts Medical School, sharanya.iyengar@umassmed.edu

Follow this and additional works at: http://escholarship.umassmed.edu/gsbs_diss

Part of the Cancer Biology Commons, Cell Biology Commons, and the Neoplasms Commons

Repository Citation


http://escholarship.umassmed.edu/gsbs_diss/796

This material is brought to you by eScholarship@UMMS. It has been accepted for inclusion in GSBS Dissertations and Theses by an authorized administrator of eScholarship@UMMS. For more information, please contact Lisa.Palmer@umassmed.edu.
INSIGHTS INTO MELANOCYTE REGENERATION AND MELANOMA INITIATION USING THE ZEBRAFISH MODEL SYSTEM

A Dissertation Presented

By

Sharanya Iyengar

Submitted to the Faculty of the

University of Massachusetts Graduate School of Biomedical Sciences, Worcester

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

October 6, 2015

Cancer Biology Program
INSIGHTS INTO MELANOCYTE REGENERATION AND MELANOMA INITIATION USING THE ZEBRAFISH MODEL SYSTEM

A Dissertation Presented
By
Sharanya Iyengar

The signatures of the Dissertation Defense Committee signify completion and approval as to style and content of the Dissertation.

Craig J. Ceol, Ph.D., Thesis Advisor

Jenna Galloway, Ph.D., Member of Committee

Michelle Kelliher, Ph.D., Member of Committee

Nathan Lawson, Ph.D., Member of Committee

Brian Lewis, Ph.D., Member of Committee

The signature of the Chair of the Committee signifies that the written dissertation meets the requirements of the Dissertation Committee.

Eric Baehrecke, Ph.D., Chair of Committee

The signature of the Dean of the Graduate School of Biomedical Sciences signifies that the student has met all graduation requirements of the school.

Anthony Carruthers, Ph.D.,
Dean of the Graduate School of Biomedical Sciences

Cancer Biology Program
October 6, 2015
ACKNOWLEDGEMENTS

I would like to thank my advisor Dr. Craig J. Ceol for giving me the opportunity to pursue my thesis research in his lab, for being a fantastic mentor and providing guidance and support, scientific and otherwise, during the past five years. I would also like to thank Melissa Kasheta and James Neiswender for being amazing work spouses, and other past and present members of the Ceol lab for friendship and advice.

I would also like to thank Dr. Eric Baehrecke for allowing me to do my first rotation in his lab and making graduate school a less intimidating place, for agreeing to be the chair of all my committees, and for being a great mentor. Thank you Dr. Arthur Mercurio, Dr. William Theurkauf, Dr. Brian Lewis, Dr. Nathan Lawson and Dr. Michelle Kelliher for reviewing my progress and helpful discussions. Thank you Dr. Jenna Galloway for taking the time to be my external examiner.

Special thanks to my parents, my brother, my boyfriend and all my friends for their constant encouragement, and my cat Quinn Iyengar who is the best late night melanocyte counting buddy a girl could ask for.
SUMMARY

During regeneration, cells must coordinate proliferation and differentiation to rebuild tissues that are lost. Understanding how source cells execute the regeneration process has been a longstanding goal in regenerative biology with implications in wound healing and cell replacement therapies. Melanocytes are pigment-producing cells in the skin of vertebrates that can be lost during hair graying, injury and disease-related depigmentation. Melanoma is an aggressive skin cancer that develops from melanocytes, and it is hypothesized that melanoma cells have properties that are similar to melanocyte stem cells.

To gain insight into melanocyte regeneration we set out to identify the source of regeneration melanocytes in adult zebrafish and the path through which progenitor cells reconstitute the pigment pattern. Using targeted cell ablation and single cell lineage-tracing analyses we identified that a majority of regeneration melanocytes arise through direct differentiation of *mitfa*-expressing progenitor cells. Concurrently, other *mitfa*-expressing cells divide symmetrically to generate additional *mitfa*-positive progenitors, thus maintaining regeneration capability. Using reporter assays and drug studies, we found that Wnt signaling gets turned on in progenitor cells during regeneration and Wnt inhibition after melanocyte ablation blocks regeneration. Based on our finding that Wnt signaling is active in differentiated melanocytes but not in the progenitor cells, we explored
the role of Wnt signaling in tumor initiation. We found that approximately half of the melanomas are Wnt silent, and overexpression of \textit{dkk1b}, a negative regulator of canonical Wnt signaling, accelerates melanoma onset.

This work defines an unappreciated contribution by direct differentiation in melanocyte regeneration and suggests a broader role for this process in the maintenance of epithelial sheets. This study also identifies a shared pathway between melanocyte progenitors and melanoma cells, which could be applicable to other cancers.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Title Page</td>
<td>i</td>
</tr>
<tr>
<td>Signature Page</td>
<td>ii</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>iii</td>
</tr>
<tr>
<td>Summary</td>
<td>iv</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>vi</td>
</tr>
<tr>
<td>List of Third Party Copyrighted Material</td>
<td>viii</td>
</tr>
<tr>
<td>List of Figures and Tables</td>
<td>ix</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>xi</td>
</tr>
<tr>
<td>Preface</td>
<td>xii</td>
</tr>
<tr>
<td>Publications</td>
<td>xiii</td>
</tr>
<tr>
<td>Chapter I Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Chapter II Identifying and characterizing the cells that mediate</td>
<td>51</td>
</tr>
<tr>
<td>melanocyte regeneration in adult zebrafish</td>
<td>52</td>
</tr>
<tr>
<td>Chapter III Role of Wnt/β-catenin signaling pathway in melanoma initiation</td>
<td>55</td>
</tr>
<tr>
<td>Chapter III Role of Wnt/β-catenin signaling pathway in melanoma initiation</td>
<td>99</td>
</tr>
<tr>
<td>Chapter III Role of Wnt/β-catenin signaling pathway in melanoma initiation</td>
<td>104</td>
</tr>
</tbody>
</table>
Chapter IV Discussion & Future Directions

Discussion 120

Future Directions 140

References 143
<table>
<thead>
<tr>
<th>Figure</th>
<th>Publisher</th>
<th>License Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure I.1</td>
<td>Nature Publishing Group</td>
<td>3733230363713</td>
</tr>
<tr>
<td>Figure I.2</td>
<td>Nature Publishing Group</td>
<td>3733230363713</td>
</tr>
<tr>
<td>Figure I.3</td>
<td>John Wiley and Sons</td>
<td>3733230659946</td>
</tr>
<tr>
<td>Figure I.4</td>
<td>Elsevier</td>
<td>3733231010511</td>
</tr>
<tr>
<td>Figure I.5</td>
<td>Mary Ann Liebert</td>
<td>Used with permission</td>
</tr>
<tr>
<td>Figure I.6</td>
<td>Nature Publishing Group</td>
<td>3733241089291</td>
</tr>
<tr>
<td>Figure I.7</td>
<td>AAAS</td>
<td>Used with permission</td>
</tr>
</tbody>
</table>
LIST OF FIGURES AND TABLES

Chapter I

Figure I.1 Melanocytes and skin pigmentation
Figure I.2 Melanocyte stem cells in the hair follicle
Figure I.3 Zebrafish pigment pattern
Figure I.4 Zebrafish pigment pattern mutants
Figure I.5 Zebrafish melanocyte development from the neural crest
Figure I.6 Schematic of the Wnt/β-catenin signaling pathway
Figure I.7 Schematic of the MC1R signaling pathway

Chapter II

Figure II.1 Melanocyte regeneration in adult zebrafish requires mitfa-expressing cells
Figure II.2 miniCoopR based ablation
Figure II.3 Unpigmented mitfa-expressing cells are present at the onset of regeneration
Figure II.4 Unmelanized mitfa-expressing cells admixed with melanocytes are not xanthophores
Figure II.5 Unpigmented mitfa-expressing cells give rise to differentiated melanocytes by direct differentiation during regeneration
Figure II.6 Unpigmented *mitfa*-expressing cells arising through miniCoopR rescue or normal ontogeny give rise to differentiated melanocytes by direct differentiation.

Figure II.7 Adult zebrafish stripes can undergo several cycles of regeneration.

Figure II.8 Some unpigmented *mitfa*-expressing cells enter the cell cycle upon melanocyte ablation.

Figure II.9 Divisions of unpigmented *mitfa*-expressing cells during melanocyte regeneration.

Figure II.10 Wnt signaling is activated during melanocyte regeneration.

Figure II.11 TOPFlash reporter signal is lost when differentiated melanocytes are ablated.

Figure II.12 Treatment of differentiated melanocytes with the Wnt inhibitor IWR1.

Figure II.13 Wnt signaling regulates melanocyte regeneration.

Table II.1 Direct differentiation and cell division of unpigmented *mitfa*-expressing cells is specific to the regeneration response.

**Chapter III**

Figure III.1 Wnt signaling is turned off in melanoma.

Figure III.2 *dkk1b* accelerates melanoma onset in zebrafish.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC</td>
<td>adenomatous polyposis coli</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP responsive-element-binding protein</td>
</tr>
<tr>
<td>DKK</td>
<td>dickkopf</td>
</tr>
<tr>
<td>DOPA</td>
<td>dihydroxyphenylalanine</td>
</tr>
<tr>
<td>DRG</td>
<td>dorsal root ganglia</td>
</tr>
<tr>
<td>EDNRB</td>
<td>endothelin receptor type B</td>
</tr>
<tr>
<td>EMT</td>
<td>epithelial to mesenchymal transition</td>
</tr>
<tr>
<td>ERBB</td>
<td>EGFR-like receptor tyrosine kinase</td>
</tr>
<tr>
<td>FUCCI</td>
<td>fluorescent ubiquitylation-based cell cycle indicator</td>
</tr>
<tr>
<td>GSK</td>
<td>glycogen synthase kinase</td>
</tr>
<tr>
<td>HFSC</td>
<td>hair follicle stem cells</td>
</tr>
<tr>
<td>hpf</td>
<td>hours post fertilization</td>
</tr>
<tr>
<td>IWR</td>
<td>inhibitor of Wnt response</td>
</tr>
<tr>
<td>LEF</td>
<td>lymphoid enhancer binding factor</td>
</tr>
<tr>
<td>LRP</td>
<td>lipoprotein receptor-related protein</td>
</tr>
<tr>
<td>mC</td>
<td>miniCoopR</td>
</tr>
<tr>
<td>MC1R</td>
<td>melanocortin-1 receptor</td>
</tr>
<tr>
<td>MITFA</td>
<td>microphthalmia-associated transcription factor</td>
</tr>
<tr>
<td>MoTP</td>
<td>(2-morpholinobutyl)-4-thiophenol</td>
</tr>
<tr>
<td>MSC</td>
<td>melanocyte stem cell</td>
</tr>
<tr>
<td>Mtz</td>
<td>metronidazole</td>
</tr>
<tr>
<td>nls</td>
<td>nuclear localization signal</td>
</tr>
<tr>
<td>NTR</td>
<td>nfsB nitroreductase</td>
</tr>
<tr>
<td>POMC</td>
<td>pro-opiomelanocortin</td>
</tr>
<tr>
<td>PTU</td>
<td>phenylthiourea</td>
</tr>
<tr>
<td>SFRP</td>
<td>secreted Fizzled-related protein</td>
</tr>
<tr>
<td>TCF</td>
<td>T cell factor</td>
</tr>
<tr>
<td>TH</td>
<td>thyroid hormone</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet radiation</td>
</tr>
<tr>
<td>WIF</td>
<td>wnt inhibitory factor</td>
</tr>
<tr>
<td>wpf</td>
<td>weeks post fertilization</td>
</tr>
</tbody>
</table>
PREFACE

The work presented in this thesis was done in collaboration with the following individuals:

Melissa Kasheta- Assisted with genotyping and cryosectioning

Arvind Venkatesan- Performed Microarray analysis (Figure III.1A)

Corrie Painter- Assisted with imaging (Figure II.2B)

We also utilized the expertise of the Morphology Core.
PUBLICATIONS

Iyengar S, Houvras Y, Ceol CJ

Iyengar S, Kasheta M, Ceol CJ

Iyengar S, Ceol CJ
Role of Wnt/β-catenin signaling pathway in melanoma initiation. In preparation.
CHAPTER I

INTRODUCTION

Stem cells and regeneration

Adult stem cells self-renew, differentiate, and persist throughout the life of a tissue to maintain homeostasis and to repair damage after injury or disease (Leblond and Walker, 1956). In some tissues there is little cellular turnover (e.g. skeletal muscle), while in others including the bone marrow, intestinal epithelium and skin, there is constant cell destruction and regeneration (Collins et al., 2005; Gros et al., 2005; Leblond and Stevens, 1948; Orkin and Zon, 2008). Continuous cell turnover or sudden tissue damage challenges stem cells to maintain a tight balance between self-renewal and differentiation in order to ensure both tissue repair and maintain the potential to regenerate in the future.

Adult stem cells reside in niches that provide microenvironments for stem cell maintenance and function (Dexter et al., 1977; Lord et al., 1975; Schofield, 1978). The ability of stem cells to self-renew and differentiate is dictated by a multitude of cues from the niche microenvironment (Morrison and Spradling, 2008; Scadden, 2006). In the skin, an empty hair follicle stem cell niche created by laser ablation is repopulated with a non stem cell population that begins to behave like a stem cell (Rompolas et al., 2013). Similarly, depleting basal cells,
stem cells in the lung, results in conversion of mature secretory cells into functional stem cells (Tata et al., 2013). In the intestine, if crypt stem cells are depleted, secretory progenitor cells can also regain stem cell status in vivo (Buczacki et al., 2013; van Es et al., 2012). Interestingly, in the airway epithelia, stem cells can also regulate their daughter progenitor cells (Pardo-Saganta et al., 2015). In this system, the daughter cells are maintained by a signal from the stem cells; loss of stem cells and not differentiated cells causes the daughter cells to differentiate. Signaling within the stem cell niche is complex and also involves stem cell-mediated remodeling and maintenance of the niche. Daughter cells have been shown to become niche cells for their parent stem cells in a number of tissues. For example, in the small intestine, the Paneth cell, a stem cell descendent, plays a critical niche role in regulating intestinal stem cells (Sato et al., 2011). Similarly, in the hair follicle, inner bulge stem cells enter the cell cycle and differentiate, before returning to the bulge where they contribute to the stem cell niche (Hsu et al., 2011). Neighboring cell populations with diverse origin and function also define the niche microenvironment. Melanocyte stem cells and hair follicle stem cells, share the niche and melanocyte stem cell activation is closely coupled to the hair cycle (Rabbani et al., 2011; Tanimura et al., 2011). Understanding the mechanisms governing stem cell quiescence, proliferation and differentiation is vital for understanding processes such as ageing and cancer development, which represent conditions in which stem cell behavior and
function is altered. This knowledge could then be exploited for the design of rational treatments, including cell-replacement therapies.

**Melanocytes and melanin**

Melanocytes are pigment-producing cells found in the epidermis of our skin that give color to our skin and hair (Lin and Fisher, 2007). They absorb ultraviolet (UV) radiation and can survive genotoxic stress, and hence protect us from the damaging effects of UV radiation (Figure I.1). Skin is the most common site of cancer, and skin color and degree of tanning response are the most useful predictors of skin cancer susceptibility (Lo and Fisher, 2014; Siegel et al., 2014). There are 73,870 estimated new cases of melanoma in 2015, which account for 4.5% of all new cancer cases but less than 2% of all skin cancers. The 5-year survival for localized melanoma is 98.3%, but that number drops down to 16.6% when the cancer has metastasized to distant sites (SEER Cancer Statistics Factsheets).

Synthesis of melanin, the pigment produced by melanocytes, begins with the conversion of tyrosine to dihydroxyphenylalanine (DOPA) by the enzyme tyrosinase (Ito and Wakamatsu, 2003). In mammals, the pathway parts downstream of DOPA and depending on the activity of the melanocortin-1 receptor (MC1R) either eumelanin (black or brown pigment) or pheomelanin (red or yellow pigment) is made (Garcia-Borron et al., 2005). Melanin synthesis has
toxic intermediates and perhaps therefore takes place in specialized lysosomes known as melanosomes within the melanocyte (Raposo and Marks, 2007). In mammals, melanin granules are transported along microtubules to the tips of the dendrites of the melanocytes and are transferred to surrounding keratinocytes. Once in keratinocytes, melanosomes are positioned on the sun side of nuclei to protect from UV (Barral and Seabra, 2004; Marks and Seabra, 2001) (More details in Figure I.7).
**Figure I.1 Melanocytes and skin pigmentation**

Left- Haematoxylin and eosin staining of normal human skin. Melanocytes (arrows) are located in the basal layer of the epidermis. The number of melanocytes mostly remains constant, and differences in pigmentation are due to the number of melanosomes and eumelanin/pheomelanin ratio in surrounding keratinocytes.

Right- Immunohistochemical staining of melanocytes by marker D5 which labels MITF.

MSCs and melanocyte regeneration in mammals

In mammals, melanocytes are found in the hair follicle and in the interfollicular epidermis. The hair follicle undergoes cyclic growth and regression, with alternating anagen (growth), catagen (regression), and telogen (rest). In mice, hair follicle stem cells (HFSCs) in the bulge region of the hair follicle mediate regeneration in each hair cycle (Fuchs, 2007), and melanocyte regeneration in the hair follicle is coupled to the hair cycle (Nishimura et al., 2002). Unpigmented, Dct-positive, Kit-independent, slow cycling mouse melanocyte stem cells (MSCs) reside in the bulge and secondary hair germ region of the hair follicle and reconstitute pigment during each cycle (Figure I.2) (Nishimura et al., 2002). In mice, differentiated melanocytes also express Dct and no MSC specific markers have been defined. Mouse MSCs are maintained in a quiescent state with global downregulation of mRNA expression (Freter et al., 2010; Osawa et al., 2005). When activated they show upregulation of a number of genes which are downregulated again during the next mid-anagen (Nishimura et al., 2005; Nishimura et al., 2010). Mouse HFSCs and MSCs activate Wnt/β-catenin signaling at the onset of anagen, and while Wnt activation in MSCs promotes their differentiation, Wnt activation in the HFSCs promotes hair follicle formation and MSC proliferation through endothelins. Canonical Wnt ligands are produced by the HFSCs, but not by the neighboring MSCs. (Rabbani et al., 2011). Inhibition of canonical Wnt signaling by β-catenin deletion in mouse melanocytes results in fewer melanocytes that fail to express the differentiation marker Mitf,
and gray hair in the third hair cycle after deletion. Downstream of Wnt signaling is microphthalmia-associated transcription factor (Mitf), the master regulator of melanocytes, and its target Bcl2, which are critical in MSC maintenance. Loss of Bcl2 in the melanocyte lineage leads to apoptosis of MSCs when they enter the dormant phase of the hair cycle, but not differentiated melanocytes (Nishimura et al., 2005). MSCs are in direct contact with HFSCs, and TGF-β secreted by the HFSCs in the niche is critical for maintaining their stemness. TGF-β signaling is activated in MSCs when they enter the dormant phase of the hair cycle and this process requires Bcl2 for cell survival. Furthermore, TGF-β type II receptor loss of function in the melanocyte lineage causes incomplete maintenance of MSCs and results in mild hair graying (Nishimura et al., 2010). B-Raf and C-Raf, and Notch1 and Notch2 receptors are also implicated in the maintenance of melanocyte stem cells as their disruption leads to hair graying (Schouwey et al., 2007; Valluet et al., 2012). MSC maintenance becomes less efficient with age and hence also leads to gray hair (Nishimura et al., 2005).

Cells that maintain melanocytes in the interfollicular epidermis have not been identified although it has been hypothesized that in volar skin the secretory portion of eccrine sweat glands may provide a niche for melanoblasts. These cells are immature and slow cycling, but can self renew and make differentiated melanocytes (Okamoto et al., 2014). Alternatively, an interfollicular population has also been suggested to be the melanocyte stem cell in epithelial sheets
based on their Kit-independent survival and their ability to differentiate into melanocytes (Kawaguchi et al., 2008).
Figure I.2 Melanocyte stem cells in the hair follicle

Dct-positive melanocyte stem cells reside in the bulge region of the hair follicle and are spatially separated from the differentiated melanocytes. They get activated during each hair cycle and differentiate into melanocytes.

Image from:
**Zebrasfish as a model system to study melanocyte biology**

The beauty and variety of vertebrate pigment patterns have fascinated biologists for many years. The pigment pattern of zebrasfish is created by different types of cells including black melanocytes, yellow xanthophores, and shiny reflective iridophores (Parichy, 2003; Rawls et al., 2001) (Figure I.3). Zebrasfish are a good model to study melanocyte biology as unlike mammalian melanocytes they retain melanin pigment and can be easily visualized and quantified. Melanocytes are dispensable for survival in the laboratory and multiple pigment mutants have been identified (Johnson et al., 1995; Kelsh et al., 1996; Parichy, 2003; Parichy et al., 2000a; Parichy et al., 2000b; Streisinger et al., 1986) (Figure 1.4). For example zebrasfish *kita* and *ednrbg1* single loss of function mutants only have about half the number of melanocytes, and *kita; ednrbg1* double mutants lack all stripe and scale melanocytes (Johnson et al., 1995). Melanocyte development from the neural crest is well studied and is conserved between fish and mammals making studies in zebrasfish translatable (Figure I.5). Zebrasfish embryos develop externally and are transparent, and many genetic manipulation techniques and drugs are available (Curado et al., 2008; Halpern et al., 2008; Kawakami, 2007; Kwan et al., 2007). Zebrasfish are relatively inexpensive and one breeding pair can provide hundreds of embryos every week.
**Figure I.3 Zebrafish pigment pattern**

The zebrafish pigment pattern is made up of alternating stripes and interstripes. The stripes contain dark melanocytes (arrowhead), iridophores and xanthophores, while the interstripes contain iridophores (i) and xanthophores (x). The morphology of the xanthophores and iridophores varies considerably between the stripes and interstripes.

Figure I.4 Zebrafish pigment pattern mutants

A few examples of adult pigment pattern mutants. Larval and adult pigment pattern mutants have helped identify genes and pathways important in melanocyte development and pigment pattern metamorphosis.

Image from:
Figure I.5 Zebrafish melanocyte development from the neural crest

sox2 is expressed in the developing ectoderm, followed by sox10 which is an early marker of specified neural crest cells of the pigment lineage. Committed melanoblasts temporally express sox10 then mitfa, followed by differentiation markers.

Image from:
Melanocyte development in zebrafish

Vertebrate pigment cells arise during development from a transient, highly migratory population of cells along the dorsal neural tube known as the neural crest (Le Douarin and Dupin, 2003). The neural crest gets specified by activation of Bmp and Notch signaling (Cornell and Eisen, 2002, 2005; Endo et al., 2003; Glavic et al., 2004), followed by Snail and Slug transcription factor-mediated induction of epithelial to mesenchymal transition (EMT) resulting in downregulation of E-cadherin and enhancement of cell migratory ability (Cano et al., 2000; Kanzler et al., 2000). Notch signaling may also be required later in the process to make melanocytes, as the zebrafish mindbomb mutant which cannot cleave Notch ligands, lacks melanocytes beyond the head region as more neural crest cells are pushed towards a neuronal fate (Itoh et al., 2003; Kelsh et al., 1996). Neural crest cells delaminate from the dorsal-most aspect of the neural tube and differentiate into fates that fall into four broad categories: pigment cells, neurons, glia, and ectomesenchymal cells. The antero-posterior position at which neural crest cells delaminate broadly defines their fate and, based on this positioning, the neural crest can be subdivided into five overlapping groups: cranial, vagal, sacral, trunk and cardiac. The neural crest in the trunk region gives rise to pigment cells, neurons, and glia among other cell types (Mayor and Theveneau, 2013). Pigment cells include committed but unpigmented melanoblasts, which then differentiate into melanocytes (Lister et al., 1999; Opdecamp et al., 1997; Raible and Eisen, 1994). In zebrafish, melanocytes
follow a dorsolateral migration pathway between the somites and epidermis, and contribute to the larval stripes. They also traverse along a ventromedial pathway, travelling along nerves to contribute to the lateral, ventral and yolk sac stripes (Dooley et al., 2013). The melanoblasts begin to express melanin before 24 hours post fertilization (hpf) even before they finish migrating to their final location. The zebrafish embryonic pigment pattern is mostly established by 48 hpf, and very few new melanocytes are added for the next 12 days (Milos et al., 1983). The larval melanocytes are slowly replaced by the adult pigment pattern from two to four weeks post fertilization (wpf) when the fish undergoes metamorphosis and adopts its adult form (Johnson et al., 1995). This consists of an early wave of stripe melanocytes, followed by more stripe melanocytes and dorsal and fin melanocytes at 3 wpf (Rawls and Johnson, 2000). As the fish grows new stripes are added to the adult pigment pattern.

**Wnt/β-catenin signaling pathway**

The Wnt/β-catenin signaling pathway is highly conserved and is critical for embryonic development, proliferation, stem cell maintenance, and disease (Bienz and Clevers, 2000; Logan and Nusse, 2004; Reya et al., 2003). In brief, Wnt ligands bind to Frizzled receptors (Janda et al., 2012) and the low-density lipoprotein receptor-related protein 5 or 6 (LRP5/6) co-receptor, leading to phosphorylation of LRP5/6 and Dishevelled (DVL) at residues required for inhibition of the β-catenin destruction complex (Pinson et al., 2000; Tamai et al., 2000).
2000). This complex also contains the scaffold axin, adenomatous polyposis coli (APC), casein kinase I (CK1), and glycogen synthase kinase 3β (GSK3β) and the E3-ubiquitin ligase β-TrCP. β-catenin accumulates in the cytoplasm and translocates to the nucleus where it interacts with members of the lymphoid enhancer binding factor (LEF) and T cell factor (TCF) family of proteins thereby activating transcription of numerous genes (MacDonald et al., 2009). Inhibitors of Wnt signaling include Dickkopf (DKK) (Glinka et al., 1998), secreted Fizzled-related protein (SFRP) (Rattner et al., 1997), and Wnt inhibitory factor (WIF) (Hsieh et al., 1999), which inhibit ligand–receptor interaction. In the presence of Wnt inhibitors or absence of Wnt ligands, β-catenin is phosphorylated first by CK1, followed by GSK3β and is ubiquitinated and degraded by the proteosome (Figure I.6) (Aberle et al., 1997; Amit et al., 2002; Liu et al., 2002).

The Wnt/β-catenin-signaling pathway is essential for neural-crest induction (Garcia-Castro et al., 2002; Lewis et al., 2004) and is one of the earliest steps in melanocyte specification. In mice, Wnt1 and Wnt3a whole body compound mutants show a marked deficiency in neural crest derivatives including pigmentation defects (Ikeya et al., 1997), and Wnt1 plays a role in melanocyte expansion and differentiation (Dunn et al., 2000). In zebrafish, expression of Wnt-1 by mRNA injection in single cell embryos, leads to an increase in the extent of neural crest markers. Labeling of neural crest cells showed that those adjacent to the Wnt1 and Wnt3a expression domain become pigment cells, while cells that
are farther away become neurons and glia. Lateral neural crest cells, which are
destined to generate neuronal cells, adopt pigment cell fates when injected with
an activated form of β-catenin. Also, expression of either a mutant form of the
transcription factor Tcf-3 or a dominant-negative Wnt-1 into medial neural crests,
which are fated to become pigment cells, resulted in a dramatic decrease in the
production of pigment cells and some of these cells generated neurons. The
dominant-negative Wnt-1 phenotype can be rescued by co-injecting β-catenin
mRNA (Dorsky et al., 1998). In the melanocyte lineage, targets of the canonical
Wnt/β-catenin signaling pathway include Mitf (Dorsky et al., 2000) and Sox10
(Lee et al., 2000; Potterf et al., 2000; Verastegui et al., 2000), which as described
in the following sections are important in determining melanocyte cell fate.
**Figure I.6 Schematic of the Wnt/β-catenin signaling pathway**

In the absence of Wnt ligand, β-catenin is recruited to the destruction complex, which leads to its ubiquitination and degradation (left). Upon binding of Wnt to Frizzled and LRP5/6, active Dishevelled recruits the destruction complex to the plasma membrane and prevents β-catenin degradation. β-catenin translocates to the nucleus and activates target genes with TCF and LEF (right).

Apart from the canonical Wnt/β-catenin pathway, which signals through the stabilization of β-catenin, the noncanonical β-catenin-independent planar cell polarity (PCP) and Wnt/calcium pathway have also been characterized. All three Wnt signaling pathways are activated by the binding of a Wnt ligand to a Frizzled receptor. One of the first indications of differences came from misexpression analysis in *Xenopus* embryos. Overexpression of Wnt1 and Wnt8, caused a duplication of the embryonic axis by canonical signaling through β-catenin. On the other hand, overexpression of XWnt4, XWnt5a, and XWnt11, caused defective convergence and extension of the body axis without affecting cell fates (Du et al., 1995; Moon et al., 1993). Among the 19 different Wnts in mammals, the majority of them transduce their signals by the Wnt/β-catenin pathway. A few ligands like Wnt5a and Wnt11 do not stabilize β-catenin, but act by inhibition of canonical Wnt/β-catenin signaling, Wnt/Calcium pathway and Wnt/PCP pathway (Ishitani et al., 2003; Ishitani et al., 1999; Torres et al., 1996).

The planar cell polarity system, was first characterized in *Drosophila*, is important for cells of many tissues to acquire cellular asymmetry to execute their physiologic functions (Adler, 1992). The PCP pathway overlaps the canonical Wnt/β-catenin signaling pathway in that it requires Frizzled and Dishevelled, but diverges downstream and does not involve the β-catenin destruction complex (Adler, 2002). The noncanonical Wnt/calcium pathway regulates calcium release inside the cell. Wnt5a or Wnt11 can activate the calcium-sensitive kinase protein
kinase C in zebrafish and xenopus (Sheldahl et al., 1999; Slusarski et al., 1997a; Slusarski et al., 1997b). Wnt/JNK signaling, and Wnt/Rho signaling pathways have also been identified and it remains to be understood whether they are pieces of the same pathway or different. For example, a PCP-specific Dsh, and Wnt5a and Wnt11 can also activate calcium signaling (Heisenberg et al., 2000; Kilian et al., 2003; Sheldahl et al., 1999; Sheldahl et al., 2003; Slusarski et al., 1997b). There is some uncertainty as to how specificity can be determined between canonical and noncanonical Wnt signaling, and as pathway specificity is usually context dependent, it may prove challenging to decipher.

**Melanocortin-1 receptor**

The melanocortin-1 receptor (MC1R) is a major determinant of pigment phenotype and the locus was first identified in mice based on coat color mutants (Rees, 2003). *MC1R* encodes a G-protein-coupled receptor, which activates adenylyl cyclase inducing cyclic AMP production (Mountjoy et al., 1992). This leads to phosphorylation of cAMP responsive-element-binding protein (CREB), which in turn activates genes including *MITF* (Levy et al., 2006). Tanning involves p53 activation in keratinocytes in response to UVR-induced DNA damage, leading to p53-mediated up-regulation of proopiomelanocortin (POMC) (Cui et al., 2007). Pro-opiomelanocortin (POMC) is the precursor for both α-MSH and ACTH, agonists of human MC1R, which cause an increase in eumelanin production through elevated cAMP levels (Barsh et al., 2000), while mutations in
POMC cause a red haired phenotype similar to mutations in MC1R (Krude et al., 1998) (Figure I.7). The agouti (Asip) gene, which encodes an antagonist of MC1R, can bind to MC1R and is responsible for pheomelanin production (Furumura et al., 1996). In zebrafish, only eumelanin is made and it is retained by the melanocyte (Mort et al., 2015).

In zebrafish, mc1r expression is restricted to eyes, skin, brain and testis (melanocytes and regions containing extracutaneous melanophores) (Selz et al., 2007). MC1R signaling in fish disperses melanin containing melanosomes, covering more surface area in the melanocyte, resulting in darkening of the fish (camouflage), while a signal from the melanin-concentrating hormone (MCH) receptor pulls the melanin to the center of the melanocyte (Braasch et al., 2007; Fujii, 2000; Richardson et al., 2008).
Figure I.7 Schematic of the MC1R signaling pathway

UVR-induced DNA damage in keratinocytes, leads to p53-mediated upregulation of POMC, produces β-endorphin and α-MSH. α-MSH stimulates MC1R on adjacent melanocytes, resulting in melanin synthesis and transfer of melanosomes to keratinocytes to protect from UVR damage.

Image from:
Mitfa

Mice with mutations at the microphthalmia (mi) locus show lack of melanocytes, small eye size, failure of secondary bone resorption, fewer numbers of mast cells, and early onset of deafness due to lack of melanocytes in the inner ear (Gruneberg, 1963; Marks and Walker, 1961; Silvers, 1979; Walker 1975). The protein encoded by this locus, Mitf, is a member of the Myc-related family of basic helix–loop–helix leucine zipper (bHLH-Zip) transcription factors (Hodgkinson et al., 1993; Moore, 1995). Its critical role in humans is evident by the pigmentary deficiencies and associated deafness due to loss of melanocytes in the inner ear seen in MITF mutant Waardenburg syndrome type IIa (WS2a) patients (Tassabehji et al., 1994; Widlund and Fisher, 2003). In zebrafish, mitfa is the master regulator of melanocytes and mitfa loss of function fish lack all melanocytes as embryos and adults (Lister et al., 1999). mitfb, a mitfa paralog following a duplication event, is coexpressed with mitfa in the retinal pigmented epithelium, but is not expressed in neural crest melanoblasts (Lister et al., 2001). Expression of mitfa in loss of function mutant zebrafish rescues melanocytes and causes some non-neural crest cells to become melanized. mitfa is expressed in melanoblasts and melanocytes at all stages of migration and differentiation (Lister et al., 1999). Even though mitfa is not required for zebrafish MSC specification (Johnson et al., 2011), mitfa expressing melanocyte progenitors associated with the dorsal root ganglia are the cells that give rise to pigmented melanocytes in the adult zebrafish (Budi et al., 2011; Dooley et al., 2013).
Interestingly, \textit{mitfa} is also expressed by another pigment cell type, the xanthophore, but is not required for them to develop (Curran et al., 2009; Lister et al., 1999; Parichy et al., 2000b). In the melanocyte lineage, \textit{mitfa} is regulated by many factors most notably Wnt signaling (Dorsky et al., 2000), α-MSH (Bertolotto et al., 1998; Busca and Ballotti, 2000), Pax3 (Potterf et al., 2000; Watanabe et al., 1998) and Sox10 (Lee et al., 2000; Potterf et al., 2000; Verastegui et al., 2000). Mitfa targets include the melanocyte differentiation genes Dct, Tyr (Bentley et al., 1994; Yasumoto et al., 1994), and Tyrp1 (Yasumoto et al., 1997).

**Foxd3**

Foxd3 plays a repressive role in melanocyte fate specification, by directly repressing the transcription of \textit{mitfa} (Curran et al., 2009). Foxd3 is expressed in neural crest cells, but not melanoblasts or melanocytes. Expression of Foxd3 in neural crest cells increases the number of glial cells at the expense of melanocytes (Thomas and Erickson, 2009).

**Pax3a**

Pax3 functions concurrently to initiate a melanocyte program by activating Mitf, while acting downstream to prevent terminal differentiation by competing with Mitf for an enhancer of Dct. These melanoblasts are committed, and poised to differentiate when repression is relieved by β-catenin (Lang et al., 2005).
**Sox10**

Sox10 is an SRY-related HMG transcription factor. In zebrafish, lack of *sox10* (colorless mutant) causes loss of pigment cells including melanocytes and deficits in components of the peripheral nervous system (Dutton et al., 2001; Kelsh and Eisen, 2000). Sox10 is needed for initiation of *mitfa* expression, and then acts as a feed forward inhibitor to block melanocyte differentiation genes. Conversely Mitfa is involved in downregulation of Sox10 to overcome its inhibitory effects and promote melanocyte differentiation (Greenhill et al., 2011).

**Kit**

Kit is a type III receptor tyrosine kinase that plays a complex role in melanocyte differentiation, survival, and migration. In mice, mutations in Kit lead to a pigment phenotype and an early failure in hematopoiesis. Homozygous Kit mutations are embryonic lethal (Geissler et al., 1988). Similar phenotypes are observed in the kit ligand *Steel (Kitl)* loss of function mice. In zebrafish *kita* loss of function mutants, melanocytes start to appear normally during development, but die and disappear by 4 dpf. *kitb*, a paralog of *kita*, is not required for melanocytes and is expressed in a non overlapping pattern with *kita*. Together the *kita* and *kitb* expression pattern in zebrafish recapitulates that of *Kit* in mice, except in primordial germ cells. (Mellgren and Johnson, 2005). No new melanocytes are made to restore the pigment pattern until metamorphosis at 14 dpf. Adult fish stripes contain only ~15% of the normal number of melanocytes (Johnson et al.,
1995; Parichy et al., 1999). The sparse like (slk) mutant encodes the zebrafish Kit ligand a (Kelsh et al., 1996), and displays a phenotype similar to that of kita mutants. Unlike in mice, zebrafish kita and slk (kitlga) mutants are viable and fertile, displaying no defects in hematopoiesis, germ cell or osteoclast development.

**Ednrb**

Endothelins (ET1, ET2, ET3) are ligands that bind to 7 transmembrane domain G-protein coupled receptors EDNRA or EDNRB. In mice, EDNRB is expressed in a subclass of premigratory and migratory neural crest cells. Mice with loss of function mutations in EDNRB or its ligand, ET3, have an almost complete lack of melanocytes (Baynash et al., 1994). On the other hand, zebrafish ednrb1 (rose) loss of function mutants have larval melanocytes that are indistinguishable from wild-type. During metamorphosis these fish make new melanocytes similar to wild-type fish from day 14-21 post fertilization, but between days 21-28 they make fewer melanocytes. Adult rose mutant fish have about half as many melanocytes as wild-type fish. These cells are arranged in stripes dorsally, whereas ventrally they are organized in spots (Johnson et al., 1995; Parichy et al., 2000a).
**Erbb3b**

*erbb3b* encodes for an EGFR-like receptor tyrosine kinase. In zebrafish embryos, *erbb3b* is expressed in neural crest cells and glia (Lyons et al., 2005) and in metamorphosing larvae, *erbb3b* is expressed in glia, melanophores and in cells of the fin. *erbb3b* mutants have normal embryonic and larval melanocytes, but have a massive deficit in the number of adult melanocytes. Erbb3b is essential during development to make precursor cells that remain quiescent in the larvae, and activated during metamorphosis to make adult melanocytes. Blastula transplantation experiments have revealed that *erbb3b* functions both autonomously and non-autonomously (Budi et al., 2011; Dooley et al., 2013; Hultman et al., 2009).

**Interactions between cells for zebrafish pigment pattern formation**

Mammals and birds have one pigment cell, the melanocyte, which produces black-brown eumelanin and red pheomelanin. In contrast, fish, reptiles, and amphibians have several pigment cell types that produce different colors (Bagnara, 1998). The striking pattern of adult zebrafish arises through the interaction of black melanocytes, yellow xanthophores, and silvery reflective iridophores. Melanocytes are found only in the dark stripes while xanthophores and iridophores are present in the stripes and interstripes (Parichy and Spiewak, 2015). Xanthophore morphology can vary; they are compact and round in the interstripes, but loose and stellate in the stripes (Mahalwar et al., 2014). Dense
silvery iridophores are found in the interstripes, while they form a bluish loose net over the melanocytes in the stripes (Frohnhöfer et al., 2013; Patterson and Parichy, 2013; Singh et al., 2014). Iridophores and xanthophores support melanocytes over a long range, but repel at short range by contact dependent depolarization (Inaba et al., 2012). During metamorphosis iridophores are capable of long-range dispersal in the skin, attracting xanthophores and directly influencing their survival, differentiation, and recruitment to the interstripes (Frohnhöfer et al., 2013; Mahalwar et al., 2014; Patterson and Parichy, 2013; Singh et al., 2014). It is still unclear whether the adult zebrafish pigment pattern follows the Turing mechanism-based model, where interactions between the pigment cells are responsible for the patterns, or depends on a prepattern specified by iridophores (Singh et al., 2015; Watanabe and Kondo, 2015a, b).

In embryos, neural crest cells differentiate into all three classes of pigment cells to generate an early larval pigment pattern of melanocyte and iridophore stripes with xanthophores distributed over the entire flank (Kimmel et al., 1995).

**Adult melanocyte precursor niche in zebrafish**

Mutants with pigment pattern defects in adult fish, but not larvae, suggest that while embryonic melanocytes develop directly from the neural crest, adult melanocytes may develop from quiescent stem cells. mitfa+ proliferative pigment cell precursors are associated with peripheral nerves and ganglia, and migrate to
the hypodermis during metamorphosis, where they differentiate into melanocytes (Budi et al., 2011). They are ErbB signaling dependent, and are absent in erbb3b loss of function picasso mutants which have normal ontogenetic larval melanocytes, but have a deficit in adult melanocytes. kita and ednrba1 loss of function mutants have mitfa+ cells comparable to wild-type zebrafish. These cells have a limited capacity to regenerate after multiple rounds of melanocyte ablation and regeneration (cycles of growth in restrictive and permissive temperatures in kita<sup>b5</sup>; csf1<sup>TrTS</sup> fish) (Budi et al., 2011). They arise from the neural crest during development and remain associated with the dorsal root ganglia (DRG) until they are activated during metamorphosis (Dooley et al., 2013); thus the DRGs may serve as their niche. Early activation is observed in embryos lacking melanocytes by injecting mitfa-morpholino. Interestingly, these cells do not require mitfa for their establishment.

As described above, larval melanocyte development from the neural crest and melanocyte generation during metamorphosis have been studied in detail. In comparison, very little is known about the MSC and melanocyte regeneration in zebrafish. Due to the lack of a MSC marker, indirect studies have been used to used to understand MSC regeneration.
Melanocyte regeneration in zebrafish

Melanocyte regeneration in zebrafish has been studied extensively using the adult caudal fin resection model or after melanocyte ablation in zebrafish larvae. Metamorphosis mutants and drugs have also been used to identify genes and pathways important in melanocyte regeneration. These studies are described briefly in the following sections.

Adult zebrafish fin resection

Fin amputation in adult zebrafish is performed by cutting the distal two-thirds of the caudal fin (Johnson and Weston, 1995). Soon after injury wound healing, blastema formation, differentiation, and outgrowth occurs (Geraudie and Singer, 1992; Haas, 1962; Santamaria and Becerra, 1991), and the fish regenerates 1-2 mm of the fin in one week (Johnson and Bennett, 1999). As the fin regenerates, melanocyte and xanthophore stripes are simultaneously reestablished in the fin (Goodrich and Nichols, 1931; Goodrich et al., 1954). Fin resection experiments in adult zebrafish have revealed a number of interesting facets about melanocyte regeneration. First, the zebrafish fin has an almost inexhaustible supply of melanocyte stem or progenitor cells. Caudal fins can be amputated and allowed to regenerate multiple times on the same fish without loss of regeneration capacity. It is hypothesized that this capacity to regenerate may be due to the importance of the fin in swimming, getting food, shoaling behavior, predator avoidance, etc. (Engeszer et al., 2004; Engeszer et al., 2008; Price et al., 2008).
Second, the majority of new melanocytes in the regenerating fin arise from unpigmented precursor cells. By taking advantage of PTU (Milos et al., 1983) treatment, which blocks synthesis of melanin, it was discovered that the source of new melanocytes are unpigmented cells (Rawls and Johnson, 2000). After resection, few preexisting melanocytes do migrate into the proximal regenerating fin, but these represent only a small minority of the new melanocytes (Rawls and Johnson, 2000). Third, mitf/kit/dct-positive melanocyte precursors are uniformly distributed in the fin, not just close to the melanocyte stripes, and are locally recruited by the amputation plane (Rawls and Johnson, 2000). The cells that mediate melanocyte regeneration in the caudal fin after resection have not been identified.

Regeneration melanocytes in the caudal fin come in two flavors: kita-dependent and kita-independent. Animals that have a loss of function mutation in kita develop ontogenetic fin melanocytes similar to wild-type fish (Johnson et al., 1995). After amputation, few preexisting melanocytes migrate into the regenerating fin similar to wild-type animals, but no de novo melanocytes appear in the kita deficient fish. Hence, kita could either be required to make melanocyte precursors during development or could be required later to recruit progenitor cells after injury. In situ hybridization experiments revealed that kita is not required for generating mitfa-positive melanoblasts, but is required during regeneration for melanocyte progenitors to proceed through a kita/dct-positive
state. Fin stripes in *kita* mutant fish are later replenished by a *kita*-independent source of de novo regeneration melanocytes (Rawls and Johnson, 2000). On closer observation, similar *kita*-dependent and *kita*-independent mechanisms make up the melanocytes in the caudal fin. In the caudal fin, *kita*-independent regeneration is a mechanism to compensate for the lack of *kita*-dependent melanocytes during the later stages of tissue repair (Rawls and Johnson, 2000).

**Melanocyte regeneration in zebrafish larvae**

As described previously, melanocytes arise from the neural crest during development (Raible and Eisen, 1994; Raible et al., 1992). Pigmented melanocytes are observed before 24 hpf and this number increases to approximately 400 melanocytes by 60 hpf. The number of larval melanocytes remains constant for about 2 weeks, after which the fish starts to undergo metamorphosis, providing a good window for assessment of the regeneration response.

**Laser ablation**

Laser ablation of melanocytes is one method to elicit a cell specific response in zebrafish larvae. Lasers have been used to treat pigmentation disorders, and for removal of tattoos and other pigments (Spicer and Goldberg, 1996). Melanin specifically absorbs laser energy, creating localized thermal damage, while surrounding cells and tissues are spared. In contrast to mammalian melanocytes,
zebrafish melanocytes retain melanin pigment and this can be used to our advantage. A 5-7 ns pulse with a 532 nm Q-switched dermatology laser has been used to ablate zebrafish larval melanocytes without causing extensive damage to the surrounding tissue or death of the fish. After treatment, melanocytes contract, appear fragmented and are extruded through the skin. Assaying regeneration in PTU treated (to block synthesis of melanin) larvae showed that new melanocytes regenerate from unpigmented precursors (Yang et al., 2004).

Taking advantage of a temperature sensitive kita loss of function mutant and reciprocal temperature shift experiments, regeneration following laser ablation of larval melanocytes was shown to be kita-dependent. But, even at permissive temperature kita activity in these fish was not enough to allow melanocyte regeneration (Yang et al., 2004). It is still unclear in this regeneration model whether kita is needed for stem cell establishment or recruitment.

Laser ablation is a useful tool that can be employed to test the role of other mutants and drugs in melanocyte regeneration. One disadvantage of this method is the small number of cells that can be ablated and hence the limited number of regenerating melanocytes that are available for analysis.
(2-morpholinobutyl)-4-thiophenol (MoTP) was first described in a screen to find drugs that affected zebrafish development (Peterson et al., 2000). MoTP treatment during development results in fish with no melanocytes and lightly pigmented retinal pigmented epithelium, suggesting that it does not only block melanin synthesis but affects melanocyte development. MoTP ablates larval melanocytes and melanoblasts, and ablation is dependent on tyrosinase activity, which converts MoTP into a toxic quinone species (Yang and Johnson, 2006). Zebrafish larvae have 460 post mitotic melanocytes by 60 hpf, but after treatment with MoTP from 14 to 72 hpf no differentiated melanocytes are visible. Melanoblasts proceed to a tyr+ and dct+ state but fail to produce the terminal differentiation marker melanin, and then the number of dct+ cells rapidly declines. When larvae are transferred to fresh egg water, melanocytes regenerate over the next four to five days. Using BrdU incorporation experiments after MoTP induced block of melanocyte development, it has been shown that almost all (97.2±2.5%) regeneration melanocytes arise through division of quiescent cells. These cells are not late stage progenitors, as they do not express dct. The regeneration melanocytes arise from unpigmented precursors that are not xanthophores or iridophores as regeneration proceeds normally in larvae that are mutant for these cells (sdy^{j9s1} and fms^{4e1} respectively) (Yang and Johnson, 2006).
Alternatively, when zebrafish larvae are treated with MoTP at 48 hpf the melanocytes appear a little lighter, fragmented and extrude from the epidermis, but are not completely cleared after 24 hours (Yang and Johnson, 2006). It is possible that MoTP does not ablate larval melanocytes but acts as a differentiation block.

4-HA

4-HA has a phenol ring structure similar to tyrosine. Tyrosine is the first substrate of tyrosinase in melanin biosynthesis. 4-HA competes with tyrosine for hydroxylation by tyrosinase and gets converted into a cytotoxic o-quinone (Naish et al., 1988). 4-HA ablates or blocks differentiation of larval melanocytes similar to MoTP, and melanocytes regenerate from unpigmented precursor cells over the next four to five days (Yang and Johnson, 2006).

After MoTP or 4-HA treatment, zebrafish larvae regenerate about 300-400 melanocytes. Approximately 50 melanocytes fail to regenerate and the majority of them are the ventral sac melanocytes, suggesting that there are either no progenitor cells in that location in the fish or they are less sensitive to loss of differentiated melanocytes (Yang and Johnson, 2006). BrdU incorporation experiments after MoTP treatment from 4-5 dpf revealed that cells incorporated BrdU within 12 hours of melanocyte death and each cell undergoes 2-4 divisions, 1.8 divisions per cell on average (Yang and Johnson, 2006).
One approach coupling melanocyte ablation with a parthenogenesis screen revealed two mutants eartha(j23e1) and julie(j24e1) (gfpt1 and skiv2l2) that have defects in melanocyte differentiation and cell proliferation, respectively, during larval melanocyte regeneration (Yang et al., 2007). A different study taking advantage of melanocyte ablation and treatment with an ErbB inhibitor AG1478 from 9-48 hpf showed that ErbB signaling is needed to establish the melanocyte stem or progenitor cells. Fish treated with the inhibitor develop larval melanocytes normally, but fail to regenerate as many melanocytes after ablation as wild-type fish or make new melanocytes during metamorphosis. This result is phenocopied by the zebrafish erbb3b loss of function picasso mutant (Hultman et al., 2009). Treatment of larvae with the inhibitor at later time points rules out a role for ErbB in stem cell recruitment, survival, proliferation, and differentiation. ErbB signaling plays a role in human embryonic stem-cell renewal (Wang et al., 2007). No deficit in regeneration after two rounds of melanocyte ablation with MoTP, with ErbB inhibition during the first round of regeneration, suggests that ErbB signaling does not play a role in zebrafish MSC renewal (Hultman et al., 2009).

Clonal analyses using transposon integration in regenerating larvae and caudal fin models

Studies using lineage-marker-bearing transposons to label clones of cells early in development have shown that all fin melanocytes including ontogenetic and
regeneration (*kita*-independent and *kita*-dependent), and xanthophores arise from the same precursor cells, while iridophores arise from a different lineage. These studies also suggest that MSCs may divide both asymmetrically to generate new melanocytes, and symmetrically to expand MSCs and leave quiescent cells behind in the regenerating fin (Tu and Johnson, 2010). Further analyses using transposon-based labeling in the caudal fin have revealed that the melanocyte-xanthophore lineage cells have their own fate restricted stem cells and these cells do not contribute to the development or regeneration of other cell types (Tu and Johnson, 2011).

In zebrafish larvae, coupling melanocyte ablation and transposon-based lineage tracing has revealed that embryonic melanocytes and larval regeneration melanocytes share common precursor cells that are segregated after the shield stage of embryo development (Tryon et al., 2011). Using the same approach it is hypothesized that the regeneration defect in *kita* loss of function mutant larvae is due to a defect in stem cell establishment and not due to a deficiency in stem cell recruitment or in the proliferation, differentiation or survival of the daughter cells. By interpretation of the rates of labeling regeneration melanocytes these larvae seem to only make half the number of stem cells that wild-type fish make and the remaining precursor cells differentiate into melanocytes instead (O‘Reilly-Pol and Johnson, 2013).
Melanocyte regeneration in adult zebrafish

Neocuproine is a drug that ablates adult zebrafish melanocytes (O’Reilly-Pol and Johnson, 2008). Its mechanism of action is not understood except that it chelates copper and melanocytes are extremely sensitive to the lack of copper. Neocuproine-mediated melanocyte ablation is not melanin or p53-dependent. Within 24 hours of treatment with neocuproine melanocytes lose their dendritic processes and the melanin begins to look contracted and punctate. In the next few days melanin appears to not be cellular and is slowly cleared. Scale melanocytes and melanocytes on the tips of fins are resistant to neocuproine treatment. Pigment pattern is restored from unpigmented precursors within four weeks of neocuproine washout (O’Reilly-Pol and Johnson, 2008).

The advantage of using neocuproine is that the ablation is specific to melanocytes and hence only melanocyte precursors are challenged to regenerate (O’Reilly-Pol and Johnson, 2008). Caudal fin amputation removes a myriad of cells types and in adults laser ablation also kills yellow xanthophores (O’Reilly-Pol and Johnson, 2008). MoTP and 4-HA fail to ablate older larval or adult melanocytes that no longer have high levels of tyrosinase (Yang and Johnson, 2006). Also, regeneration experiments in larvae are limited to two rounds of melanocyte ablation and regeneration before new melanocytes are made during metamorphosis. Neocuproine does not ablate larval melanocytes,
but prevents tyrosinase function and melanin synthesis in the zebrafish embryo (which is copper dependent) (O'Reilly-Pol and Johnson, 2008).

**Zebrafish as a model for studying melanoma**

The zebrafish has become an important model system in cancer biology with the emergence of rapid transgenesis. Site-specific recombination-based cloning allows modular assembly of promoter-coding sequence-3’ tag constructs in a plasmid with a Tol2 transposon backbone (Kwan et al., 2007). Tol2 technology enables the generation of transgenic lines by transposase-mediated insertion of the plasmid into the genome of zebrafish embryos (Kawakami, 2007). Development of heatshock, and cell type specific Cre and GAL4/UAS transgenic lines allow us to further regulate gene expression in a cell-specific and temporally restricted manner (Thummel et al., 2005). A recent study utilized Multisite Gateway and Tol2 technology in zebrafish to screen the effect of candidate genes on melanoma onset and identified a novel oncogene *SETDB1* (Ceol et al., 2011). Similarly, knockout strategies using ZFNs, TALENs and CRISPR/Cas9 are useful in identifying tumor suppressors; somatic mutagenesis makes it possible to delete essential genes specifically in melanocytes (Ablain et al., 2015; Clark et al., 2011). Large-scale zebrafish screens can assay developmental, behavioral, metabolic, proliferative and regenerative phenotypes (Rennekamp and Peterson, 2015). Advances in cell labeling like the development of Zebrabow and *ubi:switch* lines allow us to specifically label cells of interest temporally
(Mosimann et al., 2011; Pan et al., 2013). They can be coupled with new imaging strategies and provide a window for observation from the earliest stages of tumor initiation to full blown cancers to metastasis at single-cell resolution. It is also possible to observe interactions in real time between tumor cells and their microenvironment including immune cells (Feng and Martin, 2015; Heilmann et al., 2015). Advances in transplantation technology include xenotransplantation, single-cell transplantation, generation of transparent zebrafish, and rag2(E450fs) mutant zebrafish that have reduced numbers of functional T and B cells, and can engraft various cancers (Blackburn and Langenau, 2014; Tang et al., 2014; Taylor and Zon, 2009; Veinotte et al., 2014). Zebrafish rarely develop tumors spontaneously, but can be manipulated with transgenes or drugs to get benign and malignant cancers that resemble human disease and share many of the salient histopathological features (Amatruda et al., 2002).

Melanoma is an aggressive cancer arising from melanocytes. Melanocytes routinely proliferate and give rise to benign nevi or moles, but they can get transformed into tumors that grow radially and then vertically invading through the basement membrane into the dermis, followed by metastasis (Ceol et al., 2008). The incidence of melanoma is increasing, an estimated 160,000 new cases per year are diagnosed worldwide, and the overall prognosis for patients with advanced stage melanoma is poor. Some of the genes that are commonly mutated in melanoma like $BRAF^{V600E}$ and $NRAS^{Q61K}$ have been
identified, but these alone cannot explain all aspects of the disease (Davies et al., 2002). For example, \textit{BRAF}^{V600E} mutations are found not only in melanomas but also in nevi, indicating that other genetic lesions cooperate in tumor formation. Some melanomas arise from premalignant nevi, but most nevi do not give rise to melanomas (Pollock et al., 2003).

The zebrafish is an excellent model system to study melanoma biology as zebrafish melanocytes retain melanin pigment and are externally visible and quantifiable, development of melanocytes from the neural crest is well characterized and is conserved, and many pigmentation mutants are available (Ceol et al., 2008). A few zebrafish melanoma models exist (Dovey et al., 2009; Santoriello et al., 2010), the most used being one where oncogenic \textit{BRAF}^{V600E} is expressed under the control of the \textit{mitfa} promoter coupled with a \textit{p53} loss of function mutation (Patton et al., 2005). Using \textit{Tg(mitfa:BRAF}^{V600E}); \textit{p53 (lf); mitfa (lf)} fish and the miniCoopR assay which couples a wild-type copy of \textit{mitfa} to a Gateway recombination cassette, melanocytes are rescued cell autonomously and they express a gene of interest, which can be evaluated for acceleration or delay of melanoma onset. This technique has been used to rapidly distinguish genetic alterations that drive tumorigenesis from others that those arose incidentally during transformation (Ceol et al., 2011).
**Wnt/β-catenin signaling pathway and cancer**

One of the earliest connections between Wnt/β-catenin signaling and disease was made with the discovery of mouse mammary tumor virus (MMTV) activated Wnt1 (int-1) in naturally occurring mammary tumors in mice (Nusse and Varmus, 1982). This was followed by identification of germline mutations in the adenomatous polyposis coli (APC) gene in a hereditary cancer called familial adenomatous polyposis (Kinzler et al., 1991; Nishisho et al., 1991), and the subsequent finding that the APC protein interacts with β-catenin (Rubinfeld et al., 1993; Su et al., 1993). The majority of patients with colorectal cancers have inactive APC (Wood et al., 2007) and a few others have mutations in Axin2 (Lammi et al., 2004), leading to constitutive activation of the Wnt/β-catenin signaling pathway. Activation of the pathway has also been described in melanoma (Rubinfeld et al., 1997) and other cancers (Anastas and Moon, 2013; Reya and Clevers, 2005; Takeda et al., 2006). Alternatively Wnt/β-catenin signaling can also play a tumor suppressor role in cancers including melanoma (Chen et al., 2014; Chien et al., 2009).
Rationale and Objectives

The cells that mediate melanocyte regeneration in adult zebrafish are unpigmented and have a seemingly inexhaustible capacity to make new melanocytes. In spite of elegant experiments using pigmentation mutants, transposon-mediated cell labeling, and regeneration experiments in zebrafish larvae and in the adult caudal fin, the cellular source of regeneration melanocytes has not been identified. We sought to identify these cells using a targeted cell ablation approach, and to define the path through which they make new melanocytes using single cell lineage tracing. We also aimed to identify signaling pathways that are important in melanocyte regeneration using reporter assays and drugs studies. Since many of the genes that are involved in development, stem cell maintenance, and regeneration are the same genes that are misregulated in cancer, we then explored the role of the signaling pathways important in melanocyte regeneration in melanoma formation.
CHAPTER II

IDENTIFYING AND CHARACTERIZING THE CELLS THAT MEDIATE MELANOCYTE REGENERATION IN ADULT ZEBRAFISH

SUMMARY

Efficient regeneration following injury is critical for maintaining tissue function and enabling organismal survival. Cells reconstituting damaged tissue are often generated from resident stem or progenitor cells or from cells that have dedifferentiated and become proliferative. While lineage-tracing studies have defined cellular sources of regeneration in many tissues, the process by which these cells execute the regenerative process is largely obscure. Here, we have identified tissue-resident progenitor cells that mediate regeneration of zebrafish stripe melanocytes and defined how these cells reconstitute pigmentation. Nearly all regeneration melanocytes arise through direct differentiation of progenitor cells. Wnt signaling is activated prior to differentiation, and inhibition of Wnt signaling impairs regeneration. Additional progenitors divide symmetrically to sustain the pool of progenitor cells. Combining direct differentiation with symmetric progenitor divisions may serve as a means to rapidly repair injured tissue while preserving the capacity to regenerate.
INTRODUCTION

During regeneration, cells that are the source of new tissue must coordinate proliferation and differentiation to appropriately rebuild structures that are lost. The relationship between these processes impacts both the rate and extent to which new tissue is formed. Understanding the relative importance of proliferation and differentiation has been a longstanding goal in regenerative biology with implications not only in wound healing but also stem cell and other types of cell replacement therapies. Currently, there are efforts to manipulate regenerative proliferation and differentiation to improve clinical outcomes in hematopoietic stem cell transplantation, skin engraftment and other tissue restorative therapies (Ballen et al., 2013; Barrandon et al., 2012).

The relationship between proliferation and differentiation defines the mode of regeneration that occurs. In tissues where sources of cells added during regeneration are known, three modes of regeneration have been described, depending on the tissue studied and the injury model used (Poss, 2010; Tanaka and Reddien, 2011). Resident stem or progenitor cells are utilized in many tissues. Typically, these are undifferentiated cells that proliferate in response to injury to generate many descendants that differentiate to generate cells needed for repair. Hematopoietic stem cells and skeletal muscle satellite cells are exemplars of this category (Sacco et al., 2008; Sherwood et al., 2004; Weissman
and Shizuru, 2008). In other tissues, such as the mammalian liver, after partial hepatectomy, and zebrafish cardiac muscle, differentiated cells are the source (Jopling et al., 2010; Kikuchi et al., 2010; Michalopoulos, 2007). Here, remnant differentiated cells undergo dedifferentiation to enable their proliferation. The descendants generated differentiate into new cells of the same type that were lost. Lastly, transdifferentiation can occur in which a remnant cell type converts into a different cell type to replace lost cells. Whereas proliferation is critical in stem/progenitor cell and dedifferentiation modes of regeneration, it is thought to play little role during transdifferentiation. Although less common, important examples of transdifferentiation have been described, including the regeneration of the newt retina from pigmented retinal epithelial cells (Henry and Tsonis, 2010). Lineage-tracing studies have been instrumental in defining cellular sources of regeneration, yet in many cases the steps between a source cell and its differentiated descendants remain poorly understood.

To map how cells progress through the regeneration process, we have studied melanocyte regeneration in zebrafish. Melanocytes in zebrafish have emerged as a useful cell type for studying regeneration. These cells retain melanin pigment, providing a marker to distinguish differentiated cells from their progenitors. New melanocytes are made either in the context of appendage regeneration, as when the fin is resected, or following cell-specific ablation of adult stripe or embryonic melanocytes. It is clear that new melanocytes in the fin
arise from unpigmented precursors (Rawls and Johnson, 2000). Cell-specific ablations similarly implicate unpigmented precursors in regeneration of melanocytes in adult zebrafish stripes and embryos (O'Reilly-Pol and Johnson, 2008; Yang and Johnson, 2006). While some genetic regulators of melanocyte regeneration have been identified (Hultman et al., 2009; Lee et al., 2010; O'Reilly-Pol and Johnson, 2013; Rawls and Johnson, 2000, 2001; Yang et al., 2007), the source of new cells has not been defined, and the path through which source cells yield new melanocytes has not yet been described.

Here, we use a targeted cell ablation approach to define the source of regeneration melanocytes. Direct lineage determination of source cells indicates a multifaceted regeneration process involving precursor cells that directly differentiate as well as cells that divide to yield additional lineage-restricted cells. Wnt signaling is activated during melanocyte regeneration and is important for producing new melanocytes. Coupling two modes of cell replacement may be used in zebrafish and other metazoans to enable rapid cell replacement while preserving the capability to undergo multiple cycles of regeneration.
RESULTS

Ablation of mitfa-expressing cells causes failure of melanocyte regeneration

To study the mechanisms by which melanocyte regeneration occurs, we sought to identify the cells responsible for reconstituting adult zebrafish stripe melanocytes following injury. These cells are unpigmented, as regeneration occurs normally following ablation of all pigmented, differentiated melanocytes with the small molecule neocuproine (O’Reilly-Pol and Johnson, 2008). To identify the cells mediating regeneration, we used a promoter-based, cell-specific ablation approach. In this approach, a bacterial nfsB nitroreductase (NTR) gene was expressed in cells targeted for ablation. Expression of the NTR gene alone is innocuous; however, when the prodrug metronidazole (Mtz) is applied, the NTR protein processes metronidazole into toxic compounds, leading to cell death (Curado et al., 2007; Pisharath et al., 2007). Promoters were used to drive expression of the NTR gene in specific cells with the goal of finding a promoter that was active in the cells responsible for regeneration. In many cases, cells that mediate regeneration express lineage-specific genes that continue to be expressed once differentiated cells are generated (Munoz et al., 2012; Nishimura et al., 2002). With this in mind, we expressed NTR using promoters of melanocyte lineage genes. Two outcomes from this approach were predicted (Figure II.1A): 1) if the promoter were expressed only in differentiated
melanocytes, then metronidazole application would lead to melanocyte ablation followed by regeneration, or 2) if the promoter were expressed in differentiated melanocytes and cells mediating regeneration, then application of metronidazole would ablate both types of cells and regeneration would not occur.

The miniCoopR system (Ceol et al., 2011) was used to express NTR under melanocyte lineage promoters (Figures II.2A and B). miniCoopR animals are chimeric and express transgenes of interest in melanocytes and lineally related cells. Interestingly, when NTR was expressed under the control of the mitfa promoter and melanocyte-positive Tg(miniCoopR-mitfa:NTR) adults were treated with Mtz, melanocytes were ablated but regeneration did not occur (Figure II.1B). To confirm that Tg(miniCoopR-mitfa:NTR) adults were capable of regeneration, we treated these melanocyte-positive adults with neocuproine and found that melanocytes regenerated from unpigmented precursors following drug washout (Figure II.2C). These data suggest that the mitfa promoter is active not only in differentiated melanocytes but also in the cells that mediate melanocyte regeneration.
Figure II.1 Melanocyte regeneration in adult zebrafish requires mitfa-expressing cells

(A) Strategy for identifying cells that mediate melanocyte regeneration: promoter-based ablation of only differentiated melanocytes results in regeneration (left) whereas promoter-based ablation of differentiated melanocytes and cells that mediate melanocyte regeneration causes failure of regeneration (right).

(B) Adult zebrafish expressing mitfa:NTR in miniCoopR-rescued melanocytes and lineally-related cells (top). Differentiated melanocytes were ablated upon Mtz treatment (middle) but failed to regenerate after Mtz was washed out (bottom). n = 5 fish; representative images are shown.

(C) Adult p53(If) zebrafish expressing miniCoopR-mitfa:NTR (top). Differentiated melanocytes were ablated on Mtz treatment (middle), and new melanocytes regenerated after Mtz was washed out (bottom). n = 5 fish; representative images are shown. mC, miniCoopR. Scale bars = 5mm.
Figure II.2 miniCoopR based ablation

(A) Schematic of the miniCoopR vector (left). Promoters of interest were cloned into miniCoopR to drive expression of *NTR* or other genes. A recombined miniCoopR clone was injected into *mitfa(lf)* single-cell zygotes, and embryos with rescued melanocytes were selected at three days post-fertilization; scale bar = 250µM. These embryos were grown, and animals with melanocyte rescue were selected again at two months of age; scale bar = 5mm.

(B) Table describing promoters tested.

(C) Adult zebrafish expressing *Tg(miniCoopR-mitfa:NTR)* in melanocytes and cells lineally related to them (top). Stripe melanocytes were ablated when treated with neocuproine for one day (middle). After neocuproine was washed out stripe melanocytes regenerated (bottom), indicating that animals with miniCoopR-rescued melanocytes were capable of regeneration. Scale bar = 5mm. n = 3 fish; representative images are shown.
A
Clone promoter of interest into miniCoopR vector

Inject into single-cell embryos

mitfa(if)

Select embryos with melanocyte rescue

Select adults with melanocyte rescue at two months of age

B

<table>
<thead>
<tr>
<th>Promoter tested</th>
<th>Position</th>
<th>Comments</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>mitfa</td>
<td>-1869-0</td>
<td>expression in melanocytes, Mlz caused melanocyte ablation and no regeneration</td>
<td>Ceol et al, 2011</td>
</tr>
<tr>
<td>sox10</td>
<td>-3442-0</td>
<td>expression in melanocytes, Mlz caused melanocyte ablation but also death of animals before regeneration could be assessed</td>
<td>cloned from zebrafish genomic DNA</td>
</tr>
<tr>
<td>dct</td>
<td>-1434-0</td>
<td>expression in melanocytes, partial ablation of melanocytes</td>
<td>cloned from zebrafish genomic DNA</td>
</tr>
<tr>
<td>pax3a</td>
<td>-4241-0</td>
<td>no expression in melanocytes</td>
<td>cloned from DKEY 240P18</td>
</tr>
<tr>
<td>erb3b</td>
<td>-3948-0</td>
<td>no expression in melanocytes</td>
<td>cloned from DKEY-151G10</td>
</tr>
</tbody>
</table>

C

Tg(miniCoopR-mitfa:NTR)

Day 0

↓

neo (1 day)

Day 7

↓

Day 30
NTR-mediated ablation of unpigmented *mitfa*-expressing cells is *p53*-dependent

NTR induces ablation by converting Mtz into a DNA interstrand crosslinking agent, resulting in cell death (Anlezark et al., 1992; Lindmark and Muller, 1976). To determine if NTR-induced ablation proceeded through a *p53*-dependent apoptotic program, we injected miniCoopR-*mitfa:NTR* into *mitfa*(*lf*); *p53*(*lf*) embryos and reared melanocyte-positive adults. Upon treatment with Mtz, differentiated melanocytes were ablated, indicating that their death could occur independently of *p53* (Figure II.1C). However, following Mtz washout, regeneration occurred. The regenerated melanocytes were present in locations previously occupied by the ablated melanocytes, suggesting a close association between differentiated melanocytes and the unpigmented cells that give rise to new melanocytes following ablation. These data indicate that the NTR-induced ablation of cells responsible for regeneration is dependent on *p53*.

Unpigmented *mitfa*-positive cells can directly differentiate into melanocytes during regeneration

The previous experiments led us to hypothesize that a pool of unpigmented, *mitfa*-expressing cells was present near differentiated melanocytes, and these unpigmented cells were activated following ablation to give rise to new melanocytes and reconstitute the zebrafish stripe pigment pattern. To directly visualize unpigmented *mitfa*-expressing cells and track their lineages, we created
animals that expressed EGFP under the control of the mitfa promoter. An EGFP with a nuclear localization signal was used to better distinguish one cell from another as well as more accurately track mitoses. Tg(miniCoopR-mitfa:nlsEGFP) adults were generated and treated with epinephrine prior to imaging. Epinephrine promotes retrograde transport of melanosomes along microtubules within zebrafish melanocytes, leading to accumulation of these organelles in perinuclear space (Johnson et al., 1995). Melanin pigment within melanosomes absorbs ultraviolet light, and pre-treatment with epinephrine allows ultraviolet light-activated fluorophores to be readily visualized in differentiated melanocytes. Nuclei in differentiated melanocytes of Tg(miniCoopR-mitfa:nlsEGFP) adults were observed as a halo of EGFP signal surrounding the perinuclear cluster of melanosomes (Figure II.3A). In addition, nlsEGFP was observed in admixed nuclei that were smaller than those of differentiated melanocytes. Coexpression of membrane-localized mCherry-CAAX under the mitfa promoter indicated that these nuclei were present in unpigmented cells distinct from differentiated melanocytes (Figure II.3B). Another zebrafish pigment cell type, the xanthophore, also expresses mitfa (Curran et al., 2009; Lister et al., 1999). Xanthophores are mostly found in the interstripes between melanocyte stripes, but a minor population of xanthophores is also present within melanocyte stripes (Mahalwar et al., 2014). However, the unpigmented mitfa-expressing cells we observed were not xanthophores because they lacked the pteridine-based yellow pigment characteristic of this cell type (Figure II.4).
To determine if unpigmented *mitfa*-expressing cells could be responsible for regeneration, we imaged whether these cells were present at the onset of the regeneration process. Regeneration was initiated by neocuproine-induced ablation in *Tg(miniCoopR-*mitfa:*nlsEGFP)* animals (Figure II.3C) or Mtz-induced ablation in *Tg(miniCoopR-*mitfa*:NTR); *Tg(*mitfa:*nlsEGFP); *p53(lf)* animals (Figure II.3D). In both backgrounds, unpigmented *mitfa*-expressing cells were spared after ablation. Taken together, these data suggest that the unpigmented *mitfa*-expressing cells present in *Tg(miniCoopR-*mitfa:*nlsEGFP)* animals are the source of regeneration melanocytes following ablation of differentiated melanocytes. Unpigmented *mitfa*-expressing cells were also admixed with xanthophores and iridophores in interstripes, but their role in these regions is presently unknown.
Figure II.3 Unpigmented mitfa-expressing cells are present at the onset of regeneration

(A) Flank of adult zebrafish expressing miniCoopR-mitfa:nlsEGFP. Unpigmented nlsEGFP-positive nuclei (yellow arrowheads) were admixed with nuclei of differentiated melanocytes (white arrowheads).

(B) Flank of adult zebrafish coexpressing mitfa:nlsEGFP and miniCoopR-mitfa:mCherryCAAX. Unpigmented cells with nlsEGFP-positive nuclei (yellow arrowheads) were distinct from differentiated melanocytes (white arrowheads).

(C) Flank of adult zebrafish expressing miniCoopR-mitfa:nlsEGFP before (left) and after (right) neocuproine treatment. Unpigmented cells with nlsEGFP-positive nuclei (yellow arrowheads) persisted whereas differentiated melanocytes (white arrowheads) died after neocuproine treatment. n = 5 fish; representative images are shown.

(D) Flank of adult p53(lf) zebrafish coexpressing miniCoopR-mitfa:NTR and mitfa:nlsEGFP before (left) and after (right) Mtz treatment. Unpigmented cells with nlsEGFP-positive nuclei (yellow arrowheads) were spared while differentiated melanocytes (white arrowheads) were ablated following Mtz treatment. n = 3 fish; representative images are shown. Fish were treated with epinephrine prior to imaging. mC, miniCoopR; BF, brightfield. Scale bars = 100µM.
Figure II.4 Unmelanized *mitfa*-expressing cells admixed with melanocytes are not xanthophores

EGFP (left) and brightfield (right) images of the flank of an adult zebrafish expressing *Tg*(miniCoopR-*mitfa:nlsEGFP*). Unmelanized *mitfa*-expressing cells admixed with differentiated melanocytes lack the pteridine-based yellow pigment (upper insets, yellow arrowheads) seen in xanthophores (lower insets, red arrowheads) (inset scale bar = 20µM). Fish were treated with epinephrine before imaging. mC, miniCoopR; BF, brightfield. Scale bar = 100µM. Representative images are shown.
To directly link unpigmented mitfa-expressing cells and regeneration melanocytes, we performed lineage analysis following treatment with neocuproine. The flanks of Tg(miniCoopR-mitfa:nlsEGFP) animals were imaged before and after neocuproine treatment, and differentiation of new melanocytes was noted by onset of melanization. Unpigmented nlsEGFP-positive cells were tracked, and several gave rise to differentiated melanocytes without dividing (Figure II.5). In many cases these unpigmented cells were closely apposed to differentiated melanocytes. During regeneration, cells upregulated nlsEGFP expression as they began to melanize, and epinephrine-treated differentiated melanocytes showed robust nlsEGFP expression. Although nlsEGFP expression typically formed a halo around epinephrine-contracted melanosomes, in some cases it straddled opposite sides of the melanosomes (Figure II.6A). Melanocytes with such nuclear signal arose following regeneration and through normal ontogeny, and these melanocytes, along with their resident nlsEGFP signal, were readily ablated upon neocuproine treatment (Figure II.6B). During regeneration the earliest examples of direct differentiation were observed four days after neocuproine treatment. Similar results were obtained in animals expressing Tg(mitfa:nlsEGFP) in an otherwise wild-type background, indicating that observations using the miniCoopR system reflect the normal regeneration process (Figure II.6C). Thus, a subset of mitfa-expressing cells are poised to differentiate into new melanocytes following ablation.
Figure II.5 Unpigmented *mitfa*-expressing cells give rise to differentiated melanocytes by direct differentiation during regeneration

Lineage tracing of unpigmented *mitfa*-expressing cells on the flank of an adult *Tg(miniCoopR-*mitfa*:nlsEGFP)* zebrafish before and after neocuproine-mediated ablation of differentiated melanocytes. Unpigmented *mitfa*-expressing cells (yellow arrowheads) directly differentiated into pigmented melanocytes (white arrowheads). Differentiation was noted by the onset of melanization. Fish were treated with epinephrine only on day 0 prior to imaging. mC, miniCoopR; BF, brightfield. Scale bar = 50µM.
Figure II.6 Unpigmented *mitfa*-expressing cells arising through miniCoopR rescue or normal ontogeny give rise to differentiated melanocytes by direct differentiation

(A) Lineage tracing of unpigmented *mitfa*-expressing cells on the flank of an adult *Tg*(miniCoopR-*mitfa*:nlsEGFP) zebrafish before and after neocuproine ablation of differentiated melanocytes. In this example, newly formed melanocytes had nlsEGFP signal present on opposite sides of centrally-clustered melanosomes. Fish were treated with epinephrine only on day 0 prior to imaging. Scale bar = 50µM.

(B) Example of a *Tg*(miniCoopR-*mitfa*:nlsEGFP) melanocyte (white arrowheads) with nlsEGFP signal on opposite sides of contracted melanosomes. After treatment with neocuproine all nlsEGFP signal was eliminated, indicating that it was from a melanocyte nucleus. 92% (n = 25) of cells with such nuclei displayed complete elimination of nlsEGFP signal upon neocuproine treatment. Scale bar = 50µM.

(C) Lineage tracing of unpigmented *mitfa*-expressing cells on the flank of an adult wild-type zebrafish with chimeric expression of *Tg*(mitfa:nlsEGFP). In this example, an unpigmented *mitfa*-expressing cell (yellow arrowheads, day 0) is juxtaposed to an unlabeled differentiated melanocyte (white arrowheads, day 0). Following neocuproine treatment the differentiated melanocyte was ablated, and the unpigmented *mitfa*-expressing cell directly differentiated. Six of eight newly regenerated melanocytes (n = 2 fish) that were traced directly differentiated from
unpigmented *mitfa*-expressing cells. Fish were treated with epinephrine only on
day 0 prior to imaging. mC, miniCoopR; BF, brightfield. Scale bar = 25µM.
Some unpigmented mitfa-expressing cells undergo mitosis during regeneration

Since unpigmented mitfa-expressing cells can directly differentiate during regeneration, we wanted to determine if regeneration was mediated by a finite and exhaustible supply of progenitors. To begin to address this question, multiple cycles of melanocyte ablation were performed on wild-type adult fish. Individual fish were subjected to seven cycles of neocuproine-mediated ablation and regeneration. The stripe pigment pattern following the final round of regeneration was similar to that of untreated wild-type animals (Figure II.7A), indicating little if any diminution of regenerative capacity. To determine if the number of unpigmented mitfa-expressing cells is decreased following regeneration, we subjected Tg(miniCoopR-mitfa:nlsEGFP) fish to neocuproine treatment and regeneration. Tg(miniCoopR-mitfa:nlsEGFP) fish were capable of regenerating their melanocytes repeatedly, and when the flanks of Tg(miniCoopR-mitfa:nlsEGFP) animals were imaged before and after melanocyte regeneration, we observed no qualitative decrease in numbers of unpigmented mitfa-expressing cells (Figure II.7B). Together, these results led us to hypothesize that, during regeneration, unpigmented mitfa-expressing cells must be replenished from another source to compensate for the cells that directly differentiate and become pigmented.
Figure II.7 Adult zebrafish stripes can undergo several cycles of regeneration

(A) The stripe pigment pattern of an adult wild-type zebrafish before (top) and following successive cycles of ablation and regeneration (below). The number of cycles is indicated. Scale bar = 1cm.

(B) Flank of an adult $Tg(\text{miniCoopR-}\text{mitfa:nlsEGFP})$ zebrafish immediately following a first round (top) and second round (bottom) of neocuproine-induced ablation. The numbers of unpigmented $\text{mitfa}$-expressing cells at the onset of each regeneration were similar. mC, miniCoopR. Scale bar = 100µM. n = 3 fish; representative images are shown.
To determine the origin of the additional cells, Tg(miniCoopR-mitfa:nlsEGFP) animals were imaged and several unpigmented nlsEGFP cells were tracked during melanocyte regeneration. We observed that, after differentiated melanocytes were ablated, some nuclei of unpigmented mitfa-expressing cells divided to yield two mitfa-expressing daughters (Figure II.8A). The earliest examples of division were observed within two days following melanocyte ablation. To confirm that unpigmented mitfa-expressing cells could actively cycle, we adapted the fluorescent ubiquitylation-based cell cycle indicator (FUCCI) system (Sugiyama et al., 2009). This system employs two fusion proteins, mCherry-zCdt1 and mAG-zGeminin, which are expressed specifically in the G0/G1 and S/G2/M phases, respectively. We created animals coexpressing miniCoopR-ubi:mCherry-zCdt1 and mitfa:nlsEGFP transgenes or miniCoopR-ubi:AG-zGeminin and mitfa:nlsCherry transgenes. In Tg(miniCoopR-ubi:mCherry-zCdt1); Tg(mitfa:nlsEGFP) animals, nuclei of differentiated melanocytes (100%, n = 100) and unpigmented mitfa-expressing cells (100%, n = 142) were mCherry-positive (Figure II.8B), indicating they were in the G0/G1 phase of the cell cycle. In Tg(miniCoopR-ubi:AG-zGeminin); Tg(mitfa:nlsCherry) animals no differentiated melanocytes (0%, n = 300) or unpigmented mitfa-expressing cells (0%, n = 900) were AG-positive (Figure II.8C), indicating they were not actively cycling. Following neocuproine-mediated ablation in Tg(miniCoopR-ubi:AG-zGeminin); Tg(mitfa:nlsCherry) animals, some unpigmented mitfa-expressing AG-positive cells were observed (Figures
II.8C and II.8D). A low, but statistically significant, percentage of *mitfa*-expressing AG-positive cells was observed at timepoints from two through nine days following ablation. To estimate the scope of cell division, we treated wild-type zebrafish with EdU during neocuproine-mediated melanocyte ablation and regeneration (Figure II.8E). After melanocyte regeneration, we observed EdU incorporation in 25.6% (n = 224) of unpigmented Mitfa-positive nuclei (Figures II.8F and II.8G). We observed little EdU incorporation in the absence of melanocyte ablation. These data indicate that a subset of unpigmented *mitfa*-expressing cells undergo mitosis following ablation of differentiated melanocytes.

To investigate the fates of daughter cells produced by mitoses of unpigmented *mitfa*-expressing cells we further analyzed their lineages in *Tg*(miniCoopR-*mitfa:nlsEGFP*) animals. Cells were traced for approximately 10-12 days until melanin from regenerating melanocytes obscured EGFP signal. Following some mitoses, both daughter cells remained undifferentiated after regeneration was complete (Figure II.9A). In other cases, daughter cells from the same mitosis adopted different fates with one daughter differentiating and the other daughter remaining undifferentiated (Figure II.9B). The daughters that differentiated upregulated nlsEGFP expression just prior to melanization. These lineage analyses show that symmetric and asymmetric divisions of unpigmented *mitfa*-expressing cells can occur during regeneration. To quantify the relative contributions of differentiation and division, the lineages of several cells were
determined (Table II.1). Some cells neither differentiated nor divided. Of the cells that differentiated or divided (n = 55), 45.4% directly differentiated, 47.3% divided symmetrically and 7.3% divided asymmetrically (Figure II.9C). Divisions occurred between days two and seven following ablation with the median at day four. On average, the cells that directly differentiated became melanized at day 5.7±1.4 (n = 25), whereas those that melanized following asymmetric division did so at day 7.3±0.5 (n = 4). We observed no direct differentiation and little division in the absence of melanocyte ablation (Table II.1), demonstrating that both processes were not constitutive but instead part of a regeneration response. These results indicate that most regeneration melanocytes are generated by direct differentiation, and the pool of unpigmented mitfa-expressing cells is maintained primarily through symmetric divisions.

To test whether cells generated during one round of regeneration could contribute to melanocyte regeneration in subsequent rounds, we performed an EdU incorporation experiment (Figure II.9D). Fish were subjected to two cycles of melanocyte ablation and regeneration, but were treated with EdU only in the first cycle. Of melanocytes that were generated during the second cycle, 41.2% (n = 718) were EdU-positive (Figures II.9E and II.9F), indicating that descendants of cells that underwent S phase and division in the first round were source cells for melanocyte regeneration in the second round.
Figure II.8 Some unpigmented *mitfa*-expressing cells enter the cell cycle upon melanocyte ablation

(A) Flank of an adult zebrafish expressing miniCoopR-*mitfa:nlsEGFP*. Neocuproine treatment was performed for 24 hours, and hours after onset of treatment are indicated. An unpigmented *mitfa*-expressing cell undergoing mitosis is shown (yellow arrowheads). Scale bar = 100µM.

(B) Flank of an adult zebrafish coexpressing miniCoopR-*ubi:mCherry-zCdt1* and *mitfa:nlsEGFP*. Differentiated melanocytes (white arrowheads) and unpigmented *mitfa*-expressing cells (yellow arrowheads) were in the G0/G1 phase of the cell cycle. n = 100 melanocytes and n = 142 unpigmented *mitfa*-expressing cells from a total of 3 fish; representative images are shown. Scale bar = 100µM.

(C) Flank of an adult zebrafish coexpressing miniCoopR-*ubi:mAG-zGeminin* and *mitfa:nlsmCherry* before (left) and after (right) neocuproine treatment. Prior to neocuproine treatment differentiated melanocytes (white arrowheads) and unpigmented *mitfa*-expressing cells (yellow arrowheads) were not in the S/G2/M phase of the cell cycle. n = 300 melanocytes and n = 900 unpigmented *mitfa*-expressing cells from a total of 3 fish; representative images are shown. After neocuproine treatment unpigmented *mitfa*-expressing cells entered the S/G2/M phase of the cell cycle. The nucleus of an unpigmented AG-zGeminin-positive *mitfa*-expressing cell in the S/G2/M phase after neocuproine treatment is shown (yellow arrowheads). Scale bars = 100µM.
(D) Quantification of AG-zGeminin-positive unpigmented *mitfa*-expressing cells. \( n = 150 \) cells from each of three fish for neocuproine-treated group, \( n = 300 \) cells from each of three fish for no neocuproine control group. Data are shown as mean percent positive per fish ± SEM. \( P \) values calculated by Student’s \( t \)-test, * indicates \( P < 0.05 \).

(E) Timeline of the experiment. Adult wild-type zebrafish were treated with neocuproine for 24 hours to ablate differentiated melanocytes and were injected with EdU every other day (arrowheads). Control animals were injected with EdU but were not treated with neocuproine. Fish were sacrificed at day 10 and were scored for EdU incorporation.

(F) Representative images of control (top row) or neocuproine-treated (bottom row) unpigmented *mitfa*-expressing cells after melanocyte regeneration. BF scale bar = 25µM; Mitfa, EdU scale bar = 10µM.

(G) Quantification of percent EdU-positive, unpigmented Mitfa-positive cells. \( n = 103, 83 \) and 38 cells from each of three fish, respectively for neo, \( n = 20, 30 \) and 15 cells from each of three fish, respectively for w/o neo. Data are shown as mean percent positive per fish ± SEM. \( P \) value calculated by Student’s \( t \)-test. In (B) and (C) fish were treated with epinephrine prior to imaging. mC, miniCoopR; mCh, mCherry; BF, brightfield; Gem, Geminin.
Figure II.9 Divisions of unpigmented *mitfa*-expressing cells during melanocyte regeneration

Lineage tracing of unpigmented *mitfa*-expressing cells on the flank of adult *Tg(miniCoopR-*mitfa*:nlsEGFP)* zebrafish after neocuproine treatment.

(A) Symmetric division of an unpigmented *mitfa*-expressing cell (yellow arrowheads, days 0, 1, 2, 6). Neither daughter cell differentiated (yellow arrowheads, days 7, 21); scale bar = 25µM.

(B) Asymmetric division of an unpigmented *mitfa*-expressing cell (yellow arrowheads, days 0, 1, 3, 5) in which one daughter differentiated (white arrowheads, days 7, 18) and the other daughter remained undifferentiated (yellow arrowheads, days 7, 18); scale bar = 25µM.

(C) Schematic summarizing lineages of unpigmented *mitfa*-expressing cells that differentiated or divided following neocuproine-mediated ablation (dashed line). Percentages of each lineage are indicated.

(D) Timeline of the experiment. Adult wild-type zebrafish were treated with neocuproine for 24 hours to ablate differentiated melanocytes and were injected with EdU every other day until day 8 (arrowheads). After melanocyte regeneration fish were treated with neocuproine again at day 30 for 24 hours. Control animals were injected with EdU but were not treated with neocuproine. Fish were sacrificed at day 40 and were scored for EdU incorporation.
(E) Representative images of control (top row) or neocuproine-treated (bottom row) Mitfa-positive melanocytes after melanocyte regeneration. BF scale bar = 15µM; Mitfa, EdU scale bar = 10µM

(F) Quantification of percent EdU-positive, Mitfa-positive melanocytes. n = 97, 256 and 365 cells from each of three fish, respectively for neo, n = 43, 53 and 85 cells from each of three fish, respectively for w/o neo. Data are shown as mean percent positive per fish ± SEM. P value calculated by Student’s t-test. In (A) and (B) fish were treated with epinephrine only on day 0 prior to imaging. mC, miniCoopR; BF, brightfield.
Table II.1 Direct differentiation and cell division of unpigmented mitfa-expressing cells is specific to the regeneration response

Summary of lineage tracing of unpigmented mitfa-expressing cells on the flanks of adult Tg(miniCoopR-mitfa:nlsEGFP) zebrafish with or without neocuproine-induced ablation of differentiated melanocytes. *$P<1\times10^{-18}$ by Fisher’s exact test.
<table>
<thead>
<tr>
<th></th>
<th>Total cells tracked</th>
<th>Direct differentiation</th>
<th>Symmetric division</th>
<th>Asymmetric division</th>
<th>No change</th>
<th>Percent differentiation or division *</th>
<th>Fish analyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td>w neo</td>
<td>100</td>
<td>25</td>
<td>26</td>
<td>4</td>
<td>45</td>
<td>55</td>
<td>3</td>
</tr>
<tr>
<td>w/o neo</td>
<td>100</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>98</td>
<td>2</td>
<td>5</td>
</tr>
</tbody>
</table>
Wnt signaling is activated during melanocyte regeneration

To identify regulators of melanocyte regeneration, we reasoned that pathways active in melanocyte ontogeny might also be important for the regeneration process. The Wnt signaling pathway is critically involved in melanocyte development, promoting the specification of melanocytes from neural crest progenitor cells (Dorsky et al., 1998; Dunn et al., 2000; Hari et al., 2002; Hari et al., 2012; Ikeya et al., 1997; Takeda et al., 2000). To monitor Wnt activity we used the TOPFlash reporter, in which four consensus TCF/Lef binding sites are juxtaposed to a minimal c-fos promoter (Dorsky et al., 2002; van de Wetering et al., 1997). Using linearized transgenes, animals were created in which mCherry was expressed under TOPFlash control together with nlsEGFP under mitfa promoter control. The resulting Tg(miniCoopR-TOPFlash:mCherry); Tg(mitfa:nlsEGFP) adults were treated with epinephrine and imaged. mCherry signal was evident in mitfa-expressing differentiated melanocytes but absent from unpigmented mitfa-expressing cells (Figure II.10A). To confirm that the mCherry signal seen was dependent upon Wnt-responsive TCF/Lef binding sites, we used the FOPFlash variant, in which these four sites are mutated to prevent binding. Tg(miniCoopR-FOPFlash:mCherry); Tg(mitfa:nlsEGFP) fish did not express mCherry in differentiated melanocytes or in unpigmented mitfa-expressing cells. These results indicate that Wnt signaling is active in differentiated melanocytes, potentially as a means of maintaining a melanogenic program in these cells (Rabbani et al., 2011).
Figure II.10 Wnt signaling is activated during melanocyte regeneration

(A) Flank of an adult zebrafish coexpressing mitfa:nlsEGFP and miniCoopR-TOPFlash:mCherry (top) or mitfa:nlsEGFP and miniCoopR-FOPFlash:mCherry (bottom). In animals with Tg(miniCoopR-TOPFlash:mCherry), mCherry signal was evident in differentiated melanocytes (white arrowheads) but not in unpigmented mitfa-expressing cells (yellow arrowheads). No mCherry signal was observed in Tg(miniCoopR-FOPFlash:mCherry) animals. Scale bars = 100µM.

(B) Lineage tracing of unpigmented mitfa-expressing cells on the flank of an adult zebrafish coexpressing mitfa:nlsEGFP and miniCoopR-TOPFlash:mCherry after neocuproine treatment. An unpigmented mitfa-expressing cell (yellow arrowheads) beginning to express mCherry shortly before differentiation (white arrowheads). Scale bar = 50µM. In (A) fish were treated with epinephrine prior to imaging. mC, miniCoopR; BF, brightfield.
To determine if Wnt signaling is activated during regeneration, Tg(miniCoopR-TOPFlash:mCherry); Tg(mitfa:nlsEGFP) adults were immersed in neocuproine solution and unpigmented mitfa-expressing cells were tracked longitudinally. Within one day of neocuproine exposure, differentiated melanocytes were ablated and mCherry signal was lost (Figure II.11). Later, mCherry signal was observed in cells that became new melanocytes (Figure II.10B). Initiation of mCherry expression shortly preceded differentiation, as mitfa-expressing cells melanized within twenty-four hours of becoming mCherry-positive. During the course of regeneration, the cells first upregulated nlsEGFP expression, began to express mCherry, and then further upregulated nlsEGFP and mCherry expression as they melanized. Thus, Wnt signaling becomes activated during melanocyte regeneration and is closely coupled to differentiation.
Figure II.11 TOPFlash reporter signal is lost when differentiated melanocytes are ablated

Flank of an adult \( Tg(mita:nlsEGFP); Tg(miniCoopR-TOPFlash:mCherry) \) zebrafish before (top row) and after (bottom row) neocuproine treatment to ablate differentiated melanocytes. Differentiated melanocytes (arrowheads) expressed nlsEGFP and mCherry, but both signals were lost in these cells within one day of neocuproine treatment. Fish were treated with epinephrine before imaging. mC, miniCoopR; mCh, mCherry; BF, brightfield. Scale bar = 100µM. n = 3 fish; representative images are shown.
Inhibition of Wnt signaling compromises melanocyte regeneration

To investigate the functional significance of Wnt activation during regeneration, we treated fish with an inhibitor of Wnt signaling after ablation of differentiated melanocytes. IWR-1, a tankyrase inhibitor, has been shown to downregulate Wnt signaling in zebrafish and other systems (Chen et al., 2009; Huang et al., 2009). IWR-1 downregulated TOPFlash:mCherry signal in melanocytes, indicating it can act in this lineage to inhibit Wnt signaling (Figure II.12A). To inhibit Wnt signaling during regeneration, wild-type fish were treated with neocuproine for one day then, after washout, were treated with IWR-1 (Figure II.13A). The effect of IWR-1 during regeneration was measured by quantifying the number of differentiated melanocytes present at various time points during and upon completion of regeneration. Treatment with IWR-1 led to a reduction in the number of regeneration melanocytes: while an average of nearly 80% regeneration was observed in control animals by fifteen days post-ablation, only 35% regeneration occurred in IWR-1 treated fish (Figures II.13B and II.13C). The effects of Wnt inhibition were specific to the regeneration process, as treatment of age-matched wild-type animals with IWR-1 in the absence of neocuproine-mediated ablation caused no change in the number of differentiated melanocytes (Figures II.12B and II.12C). Taken together, these data indicate that Wnt signaling plays an important role in generating differentiated melanocytes during regeneration.
Figure II.12 Treatment of differentiated melanocytes with the Wnt inhibitor IWR-1

(A) Flank of an adult zebrafish expressing Tg(miniCoopR-TOPFlash:mCherry) before (top row) and after (bottom row) treatment with IWR-1. Treatment with IWR-1 for 24 hours reduced mCherry signal in differentiated melanocytes. mC, miniCoopR; BF, brightfield. Scale bar = 100 µM. n = 3 fish; representative images are shown.

(B) Representative images of adult wild-type zebrafish treated with DMSO alone or IWR-1 in DMSO without prior neocuproine-induced melanocyte ablation. Scale bar = 5mm.

(C) Quantification of melanocytes (see Experimental Procedures) expressed as a ratio of melanocytes present at the specified time point to the number of melanocytes present initially. Data are shown as mean ± SEM. P values calculated by Student’s t-test, Day 7 P = 0.43, Day 10 P = 0.95, Day 12 P = 0.82, Day 15 P = 0.59. n = 2 fish. In (A) and (B) fish were treated with epinephrine before imaging.
A

$T_g(mC-TOPFlash:mCherry)$

Pre IWR-1

Post IWR-1

B

w/o IWR-1

w/ IWR-1

Day 0  Day 7  Day 10  Day 15

C

Percent initial melanocytes

Days post-treatment
Figure II.13 Wnt signaling regulates melanocyte regeneration

(A) Timeline of the experiment. Adult wild-type zebrafish were treated with neocuproine for 24 hours to ablate differentiated melanocytes and were then treated with the Wnt inhibitor IWR-1 for 14 days. Control animals were treated with neocuproine and then DMSO alone. Fish were scored for regeneration melanocytes at 7, 10, 12 and 15 days (arrowheads) after neocuproine treatment. 

(B) Representative images of control (top row) or IWR-1-treated (bottom row) animals during melanocyte regeneration. Scale bar = 500µM

(C) Quantification of melanocyte regeneration. Data are shown as mean ± SEM. *P values calculated by Student’s t-test, *P<0.05, Day 7 P=0.36. In (B) fish were treated with epinephrine prior to imaging.
DISCUSSION

Our studies have demonstrated that mitfa-expressing cells are required for regeneration of zebrafish melanocytes. Direct lineage determination of unpigmented mitfa-expressing cells following injury indicates a poised mechanism of regeneration, one that couples direct differentiation of extant progenitor cells with divisions of additional cells that replenish the progenitor population. Since Morgan’s incisive discussion of the topic (Morgan, 1901), modes of regeneration have been categorized into those that utilize cell division and those in which regeneration occurs without cell division. However, a mechanism lacking any cell division, e.g. one solely dependent on direct or transdifferentiation, would likely result in impaired regeneration as the pool of source cells is expended. By coupling direct differentiation and division of lineage-restricted cells, regeneration of melanocyte stripes solves this problem, resulting in an extensive and perhaps unlimited capacity to replace this tissue.

This mechanism of regeneration is notable in two respects. First, to our knowledge, a prominent role for direct differentiation of progenitor cells has not been described in other systems. Lineage-tracing experiments have shown that regenerated cells can be derived from the progeny of tissue-resident stem or progenitor cells, the progeny of cells that have undergone dedifferentiation or, in some cases, from cells that have transdifferentiated. The extent of direct
differentiation may reflect the high ratio of progenitor to differentiated cells. In systems where few progenitors must give rise to many differentiated cells, division may be more prevalent. However, in systems where a surfeit of progenitor cells is present, direct differentiation may have a predominant role. Second, the presence of a large quantity of undifferentiated precursor cells is unexpected. Enough precursor cells are present to enable most new melanocytes to arise via direct differentiation. Stripe melanocytes in the fish are important for predator avoidance and fish-to-fish recognition (Engeszer et al., 2004; Engeszer et al., 2008; Price et al., 2008), and it may be advantageous to regenerate these cells rapidly following injury. A bias toward direct differentiation from an abundant progenitor pool may enable rapid regeneration of critical tissues.

Several studies have examined how new melanocytes are generated in zebrafish following injury or through normal development. Regeneration of melanocytes following amputation of the zebrafish fin has been extensively studied, but the cells that give rise to new fin melanocytes have not yet been identified. It is clear that the source cells are unpigmented (Rawls and Johnson, 2000), so divisions of differentiated cells do not appear to be involved. Furthermore, clonal analyses indicate they are lineage-restricted (Tu and Johnson, 2011), arguing against transdifferentiation as a mechanism of regeneration. Two classes of progenitor cells have been found in the zebrafish
fin, one class that can divide between one to three times before differentiating and another class whose cell division is less limited (Tu and Johnson, 2010). It is possible that the *mitfa*-expressing cells that give rise to new melanocytes in the flank are similarly used in fin melanocyte regeneration. However, the context of regeneration in the two injury models is different: neocuproine-mediated injury specifically ablates one cell type whereas fin amputation involves replacement of several cell types following formation of a blastema. The blastema provides an environment in which proliferative signals abound, and may result in greater proliferation of melanocyte precursors during fin regeneration. That robust proliferation can occur during fin regeneration is suggested by the excess BrdU-positive melanocytes that are generated when constitutively active Ras is expressed during fin regrowth (Lee et al., 2010). Our results suggest that at least a subset of *mitfa*-expressing cells have the capacity to proliferate during regeneration. If the same cells are the source of regeneration melanocytes in both the flank and fin, the extent of proliferation may be dependent on the regeneration context. In the flank where several cells contribute to replace melanocytes the need for proliferation is low. In the fin, fewer cells are likely to contribute to regeneration and the amount of proliferation may be correspondingly increased. It is also notable that melanocyte regeneration can occur in zebrafish embryos, and nearly all new melanocytes arise through proliferation (Yang and Johnson, 2006), suggesting that fewer sources of new melanocytes are present at the time of injury. In ontogenetic development, a
small number of mitfa-expressing cells associated with the dorsal root ganglia (DRG) of zebrafish larvae are proposed to generate adult melanocytes that arise during metamorphosis (Dooley et al., 2013). Given their intrinsic similarities, it is possible that the DRG-associated mitfa-expressing cells establish the pool of unpigmented mitfa-expressing cells that are essential for melanocyte stripe regeneration. In future studies it will be important, through transplantation and other approaches, to determine the potential of the unpigmented mitfa-expressing cells and whether they are involved in melanocyte regeneration at other anatomic locations or can functionally substitute for developmental progenitors. Identifying signals that govern melanocyte regeneration is also critical. Similar to what we have shown in zebrafish stripe regeneration, Wnt signaling controls differentiation of mammalian follicular melanocyte stem cells (Rabbani et al., 2011). It will be interesting to determine additional developmental pathways that are utilized during regeneration and which, if any, initiate the regenerative process.

A poised mechanism of regeneration may enable rapid replacement of a critical cell type in other systems. For example, during follicle regrowth in mammals, epidermal hair follicle stem cells adopt different fates, depending on their position in the niche (Rompolas et al., 2013). Those closest to the growing follicle exit their niche in the bulge region of the permanent follicle region and become transit-amplifying cells, whereas those more distal retain stem cell
identity. The situation with mammalian melanocyte stem cells (MSCs) is less clear. MSCs can proliferate extensively during hair follicle regrowth (Nishimura et al., 2002). However, under certain circumstances such as UV-induced interfollicular epidermal injury, MSCs can prioritize differentiation over stem cell maintenance (Chou et al., 2013). By employing cells predisposed to differentiation, poised regeneration could provide a means of repairing vital tissues and cell types with minimal delay.
EXPERIMENTAL PROCEDURES

Fish stocks and husbandry

Fish stocks were maintained at 28.5°C on a 14L:10D light cycle (Westerfield, 2007). The wild-type strain used was AB. Alleles used in this study were mitfa^{w2} (Lister et al., 1999) and p53^{zdf1} (Berghmans et al., 2005).

DNA constructs

sox10, dct, pax3a and erbb3b promoters were PCR amplified from zebrafish genomic DNA, the TOPFlash reporter promoter containing Tcf/Lef binding sites was PCR amplified from plasmid TOPdGFP and cloned into pDONRP4P1r (Life Technologies). The NTR and NTRmCherry from plasmid UNM, mCherry-zCdt1 and mAG-zGeminin from plasmids ZD:mCherry-zCdt1(1/190)/pT2KXIGΔin and ZB:mAG-zGeminin(1/100)/pT2KXIGΔin, respectively, were PCR amplified and cloned into pDONR221 (Life Technologies). Oligonucleotides used are described in Supplemental Experimental Procedures. To create the FOPFlash reporter promoter, a gBlock (IDT) containing FOPFlash (TOPFlash with mutated Tcf/Lef binding sites (van de Wetering et al., 1997) was used for cloning into pDONRP4P1r (Life Technologies). The construction of miniCoopR and pENTRP4P1r-mitfa was previously described (Ceol et al., 2011). pENTR5′_ubi (Mosimann et al., 2011) and the following components of the Tol2Kit (Kwan et al., 2007) were used: pME-nlsEGFP, pME-mCherryCAAX, pME-mCherry, p3E-
**polyA, pDestTol2pA2, pCS2FA-transposase.** Using the entry clones described above, multisite Gateway cloning (Life Technologies) was used to make the following constructs for injection (a polyA signal was used as the 3’ element in all cases): miniCoopR-\textit{mitfa}:NTR, miniCoopR-\textit{mitfa}:NTRmCherry, miniCoopR-sox10:NTRmCherry, miniCoopR-dct:NTRmCherry, miniCoopR-\textit{mitfa}:nlsEGFP, miniCoopR-\textit{mitfa}:mCherryCAAX, miniCoopR-\textit{ubi}:mCherry-zCdt1, miniCoopR-\textit{ubi}:mAG-zGeminin, miniCoopR-\textit{TOPFlash}:mCherry, miniCoopR-\textit{FOPFlash}:mCherry, Tol2pA2-\textit{mitfa}:nlsEGFP, Tol2pA2-\textit{mitfa}:nlsmCherry. For metronidazole-mediated ablation either miniCoopR-\textit{mitfa}:NTR or miniCoopR-\textit{mitfa}:NTRmCherry transgene was used.

**Microinjection and transgenic fish**

For transposon-mediated integration, 25 picograms of a construct was injected along with 25 picograms Tol2 transposase mRNA into one-cell embryos. For injections of two constructs, constructs were linearized and 25 picograms of each coinjected into embryos. When injected into zebrafish embryos, linearized transgenes cosegregate such that transgenic cells in resulting chimeric animals contain both transgenes (Langenau et al., 2008). The miniCoopR system was used to generate transgenic animals (Ceol et al., 2011). Using this system, transgenes were juxtaposed to a \textit{mitfa} minigene in the miniCoopR vector, and
the resulting constructs injected into melanocyte-deficient mitfa(If) mutant animals. The mitfa minigene is capable of rescuing melanocytes in mitfa(If) mutants cell autonomously, causing any transgene juxtaposed to the mitfa minigene to be present in rescued melanocytes and cells lineally related to them.

Lineage analysis

Adult zebrafish were treated with neocuproine in a beaker for 24 hours and then kept individually in tanks after neocuproine was washed out. Prior to imaging, fish were anesthetized with 0.17 mg/ml tricaine and were placed on their sides in a plastic petri dish. The same locations on the fish were identified using patterns of miniCoopR rescue as a landmark and were imaged three times or once daily before and after neocuproine treatment. Fish were viewed with a Leica M165FC stereomicroscope and images captured with a Leica DFC400 camera. The fish were allowed to recover in fish water.

EdU incorporation assay and histological methods

Wild-type fish injected with EdU were sacrificed, fixed for two hours in freshly prepared 4% PFA at room temperature, and 14μm cryosections were cut. The sections were stained with Mitfa primary antibody (1:100) (Ceol et al., 2011) followed by Alexa Fluor 488 secondary antibody (Life Technologies). EdU development was performed (Click-iT EdU Alexa Fluor 594 Imaging Kit; Life
Technologies), and sections were stained with DAPI and mounted (Fluoromount G; SouthernBiotech).

**Drug treatments**
Metronidazole (Sigma-Aldrich) was dissolved to a final concentration of 5mM in fish water containing 0.02% DMSO. Adult zebrafish were treated for three cycles of three days on followed by three days off drug. Neocuproine (Sigma-Aldrich) was used at 750nM in fish water containing 0.0075% DMSO. Neocuproine treatment was performed as described previously (O’Reilly-Pol and Johnson, 2008). Epinephrine (Sigma-Aldrich) was used at 1mg/mL in fish water, and treatments were performed for ten minutes immediately prior to imaging. IWR-1 (Sigma-Aldrich) was used at 10µM in fish water containing 0.1% DMSO. 10µL EdU (Life Technologies) at 10mM in 0.9X PBS containing 0.1% DMSO was injected intraperitoneally at indicated timepoints.

**Imaging and quantitative analysis**
Whole-fish images were captured using a Nikon D90 camera equipped with SB-R200 wireless remote speedlights. Fish with fluorescent markers were viewed with a Leica M165FC stereomicroscope or a Leica DM550B compound microscope and images captured with Leica DFC400 or DFC365FX cameras, respectively. Brightfield images were adjusted for contrast and color balance for clarity. Sections were viewed with a Leica DM550B compound microscope and
images were captured with DFC365FX or DMC2900 cameras. To quantify the effect of IWR-1 on melanocyte regeneration, flanks of fish were imaged and melanocytes counted on the left side center stripe of each fish in a rectangular region delimited as a 5.8mm x 1.2mm window with its left boundary 1mm posterior to the edge of the operculum. Percent regeneration was calculated as a ratio of the number of melanocytes within the region at the specified time point to the number of melanocytes within the region prior to neocuproine treatment. Student’s t-tests were performed using Prism 6.
CHAPTER III

ROLE OF WNT/β-CATENIN SIGNALING PATHWAY IN MELANOMA INITIATION

We recently identified that Wnt/β-catenin signaling is active in differentiated melanocytes but not in melanocyte progenitor cells (Chapter II, Figure II.10A). It is also known that Wnt expression in zebrafish neural crest cells causes them to differentiate into melanocytes (Dorsky et al., 1998; Dorsky et al., 2002), while in mice, Wnt activation in MSCs promotes their differentiation (Rabbani et al., 2011). It has long been hypothesized that many tumor cells adopt embryonic or adult stem cell like properties in order to bypass senescence, become more invasive, metastasize or acquire resistance to therapy (Reya et al., 2001). This prompted us to explore Wnt signaling status in melanoma.

We first used microarray analysis to compare the similarities and differences in Wnt signaling activity between differentiated melanocytes and melanoma cells from Tg(mitfa:BRAF<sup>V600E</sup>); p53<sup>lf</sup>; alb<sup>lf</sup>; Tg(mitfa:EGFP) zebrafish. In these fish oncogenic BRAF<sup>V600E</sup> is expressed under the control of the mitfa promoter and is coupled with a p53 loss of function mutation (Patton et al., 2005). These fish develop nevi and then melanomas, and their melanocytes and tumor cells are labeled with EGFP. The fluorescence label and lack of
melanin pigment makes it easy to isolate cells by FACS for RNA extraction and further analysis. Overall, we found that negative regulators of Wnt signaling including axin1 and 2, glycogen synthase kinase 3 beta (GSK3B) and Wnt inhibitory factor (WIF) are upregulated in melanoma as compared to melanocytes, and conversely, positive regulators of Wnt signaling like β-catenin are upregulated in melanocytes as compared to melanomas (Figure III.1A).

We then decided to assay Wnt status longitudinally in melanocytes and melanoma cells. To monitor Wnt activity we used the TOPFlash reporter, in which four consensus TCF/Lef binding sites are juxtaposed to a minimal c-fos promoter (Dorsky et al., 2002; van de Wetering et al., 1997). We coupled this to the miniCoopR system where a wild-type copy of the mitfa melanocyte specification factor (mitfa minigene) is juxtaposed to a Gateway recombination cassette into which any gene of interest can be recombined. miniCoopR rescues melanocytes cell autonomous in mitfa (lf) animals. Because they are physically coupled to the mitfa rescuing minigene, recombined genes are expressed in rescued melanocytes, some of which will transform and develop into tumors in Tg(mitfa:BRAFV600E); p53 (lf); mitfa (lf) animals (Ceol et al., 2011). Tg(mitfa:EGFP) was used to label melanocytes and alb (lf) was used to visualize fluorescence more clearly. Animals were created in which mCherry was expressed under TOPFlash control.
Figure III.1 Wnt signaling is turned off in melanoma

(A) Heat-map of Wnt signaling pathway genes in zebrafish melanomas (n=5 tumors) compared with control melanocytes (n=3 fish) from Tg(mitfa:BRAF^{V600E}); p53 (lf); alb (lf); Tg(mitfa:EGFP) zebrafish.

(B) Flank of an adult Tg(miniCoopR-TOPFlash:mCherry); Tg(mitfa:EGFP); Tg(mitfa:BRAF^{V600E}); p53 (lf); alb (lf) zebrafish before (top row) and after (bottom row) melanoma onset. Fish initially expressed EGFP and mCherry, but mCherry signal was lost in tumors in 5/11 fish. Nearby melanocytes continued to express both EGFP and mCherry (arrowheads). mC, miniCoopR; representative images are shown.
A

<table>
<thead>
<tr>
<th>Melanoma</th>
<th>Melanocyte</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKT3</td>
<td></td>
</tr>
<tr>
<td>PSFN1</td>
<td></td>
</tr>
<tr>
<td>AXIN1</td>
<td></td>
</tr>
<tr>
<td>PIN1</td>
<td></td>
</tr>
<tr>
<td>MVP</td>
<td></td>
</tr>
<tr>
<td>WIF1</td>
<td></td>
</tr>
<tr>
<td>GSK3B</td>
<td></td>
</tr>
<tr>
<td>CSNK1A1</td>
<td></td>
</tr>
<tr>
<td>PTPRA</td>
<td></td>
</tr>
<tr>
<td>AXIN2</td>
<td></td>
</tr>
<tr>
<td>TSHB</td>
<td></td>
</tr>
<tr>
<td>DKK2</td>
<td></td>
</tr>
<tr>
<td>DKK1</td>
<td></td>
</tr>
<tr>
<td>SFRP1</td>
<td></td>
</tr>
<tr>
<td>FSTL1</td>
<td></td>
</tr>
<tr>
<td>CTNNB1</td>
<td></td>
</tr>
</tbody>
</table>

B

- **Tg(mitfa:EGFP)**
- **Tg(mC-TOPFlash:mCherry)**

Melanocytes

Melanoma
The resulting \( Tg(\text{miniCoopR-TOPFlash:mCherry}); Tg(\text{mitfa:EGFP}); Tg(\text{mitfa:BRAF}^{V600E}); p53 \text{ (lf)}; alb \text{ (lf)} \) adults were imaged. In the dorsal region of the zebrafish, between the head and the anal fin, both EGFP and mCherry signal was present (Figure III.1B, top row). To determine the activity of Wnt signaling during melanoma initiation, the fish were tracked longitudinally once a week from two months till tumor onset. All fish started out with mCherry expression but during the course of tumor initiation mCherry signal was lost in approximately half of the lesions (5/11 fish) (Figure III.1B, bottom row). Interestingly, the fish that lost mCherry expression in the lesions still expressed it in other \text{mitfa}-expressing cells on the fish (Figure III.1B, arrowheads), indicating that loss of Wnt activity was specific to the tumor cells.

To investigate the functional significance of loss of Wnt signaling during melanoma initiation, we injected \textit{dkk1bGFP}, a negative regulator of Wnt signaling fused to GFP (Stoick-Cooper et al., 2007), into embryos to create \( Tg(\text{miniCoopR-mitfa:dkk1bGFP}); Tg(\text{mitfa:BRAF}^{V600E}); p53 \text{ (lf)} \) animals (Figure III.2A) (Ceol et al., 2011). Dkk1 prevents activation of the Wnt/\( \beta \)-catenin signaling pathway by binding to LRP5/6 (Bafico et al., 2001; Mao et al., 2001; Semenov et al., 2001). Surprisingly, expression of \textit{dkk1b} under the control of the \textit{mitfa} promoter did not block melanocyte development, perhaps because this blocks Wnt signaling after the melanocyte is already specified.
**Figure III.2 dkk1b accelerates melanoma onset in zebrafish**

(A) Schematic of the miniCoopR assay used to make chimeric animals for assaying melanoma onset

(B) Representative melanomas in *Tg(mitfa:BRAF^{V600E}); p53 (lf); mitfa (lf)* fish expressing miniCoopR-EGFP or miniCoopR-dkk1bGFP

(C) Melanoma-free survival curves for *Tg(mitfa:BRAF^{V600E}); p53 (lf); mitfa (lf)* zebrafish injected with miniCoopR-EGFP (*n* = 126) or miniCoopR-dkk1bGFP (average of 2 independent experiments, *n* = 89). *P* = 7.99 × 10^{-15}, logrank chi-squared test.
A

miniCoopR with gene of interest

\[ \text{Inject into single-cell embryos} \]

\[ Tg(mC-mitta:BRAF^{V600E}); p53(−/−); mitta(−/−) \]

Select embryos with melanocyte rescue

Select adults with melanocyte rescue at two months of age

Assay weekly for melanoma onset

B

miniCoopR-EGFP

miniCoopR-dkk1bGFP

C

Percent melanoma-free

\[ \text{Age (weeks)} \]

miniCoopR-EGFP (n=126)

miniCoopR-dkk1bGFP (n=89)
We have previously shown that blocking Wnt signaling with IWR-1 does not affect differentiated melanocytes (Figures II.12B and II.12C). Overexpression of dkk1b under the mifa promoter lead to acceleration of melanoma formation and the median onset was 11 weeks as compared to 15 weeks for the EGFP expressing control fish ($P = 7.99 \times 10^{-15}$, logrank chi-squared test; Figures III.2B and C).

It is important to note that it is possible that the cell of origin of melanoma is not the differentiated melanocyte but the unpigmented mitfa-expressing progenitor cell and hence a proportion of tumors do not express TOPFLASH. But if that was the case we should still see some mCherry expression from the adjacent differentiated melanocytes instead of complete Wnt silence. There could also be downregulation of Wnt signaling in the surrounding melanocytes by the progenitor cell that was transformed and would be interesting to explore in the future. Taken together, these data indicate that Wnt signaling plays a role in melanoma initiation, perhaps in part due to the cells acquiring progenitor cell like properties. Future directions could include assaying the invasion, transplantation and drug resistance capabilities of these tumors.
EXPERIMENTAL PROCEDURES

Fish stocks and husbandry

Fish stocks were maintained at 28.5°C on a 14L:10D light cycle (Westerfield, 2007). The wild-type strain used was AB. Alleles used in this study were mitfa\textsuperscript{w2} (Lister et al., 1999), p53\textsuperscript{edfl} (Berghmans et al., 2005), alb\textsuperscript{b4} (slc45a2\textsuperscript{b4}) (Dooley et al., 2013; Streisinger et al., 1986) and Tg(mitfa:BRAF\textsuperscript{V600E}) (Patton et al., 2005).

DNA constructs

The TOPFlash reporter promoter containing Tcf/Lef binding sites was PCR amplified from plasmid TOPdGFP and cloned into pDONRP4P1r (Life Technologies). dkk1bGFP was PCR amplified from plasmid hsdkk1GFP (Stoick-Cooper et al., 2007) and cloned into pDONR221. The construction of miniCoopR and pENTRP4P1r-mitfa was previously described (Ceol et al., 2011). The following components of the Tol2Kit (Kwan et al., 2007) were used: pME-EGFP, pME-mCherry, p3E-polyA, pCS2FA-transposase. Using the entry clones described above, multisite Gateway cloning (Life Technologies) was used to make the following constructs for injection (a polyA signal was used as the 3' element in all cases): miniCoopR-TOPFlash:mCherry, miniCoopR-EGFP and miniCoopR-dkk1bGFP.
**Microarray**

Melanomas (n=5 animals) or normal scales (n=3 animals) from the dorsal region of \( Tg(mitfa:BRAF^{V600E}); \ p53 \ (lf); \ alb \ (lf); \ Tg(mitfa:EGFP) \) zebrafish were dissected or plucked. Cells were dissociated using Liberase TH and the reaction was stopped using FBS. Melanoma cells or melanocytes were sorted using EGFP positivity; scales from \( Tg(mitfa:BRAF^{V600E}); \ p53 \ (lf); \ alb \ (lf); \ Tg(mitfa:EGFP); \ mitfa \ (lf) \) fish, lacking melanocytes, were used as negative controls to make flow cytometry gates. From these cells, RNA was isolated, and was amplified to make cDNA using the Nugen Ovation RNA Amplification system V2, which was then hybridized to a 385K microarray (NimbleGen 071105Zv7EXPR). Pathway analysis was done using GSEA with the comparative gene expression values as a rank ordered gene list.

**Microinjection and transgenic fish**

For transposon-mediated integration, 25 picograms of a construct was injected along with 25 picograms Tol2 transposase mRNA into one-cell embryos. The miniCoopR system was used to generate transgenic animals (Ceol et al., 2011). Using this system, transgenes were juxtaposed to a \( mitfa \) minigene, which when injected into melanocyte-deficient \( Tg(mitfa:BRAF^{V600E}); \ p53 \ (lf); \ mitfa \ (lf) \) or \( Tg(mitfa:EGFP); \ Tg(mitfa:BRAF^{V600E}); \ p53 \ (lf); \ mitfa \ (lf); \ alb \ (lf) \) mutant animals rescued melanocytes cell autonomously, causing any transgene juxtaposed to the \( mitfa \) minigene to be present in rescued melanocytes and cells lineally related...
to them. Transgenic animals were selected based on the presence of rescued melanocytes or expression of fluorescent marker at 48 hours post fertilization.

**Imaging**

Prior to imaging, fish were anesthetized with 0.17 mg/ml tricaine and were placed on their sides in a plastic petri dish. The same locations on the fish were identified using patterns of miniCoopR rescue as a landmark. Whole-fish images were captured using a Nikon D90 camera equipped with SB-R200 wireless remote speedlights. Fish with fluorescent markers were viewed with a Leica M165FC stereomicroscope or a Leica DM550B compound microscope and images captured with Leica DFC400 or DFC365FX cameras, respectively. Brightfield images were adjusted for contrast and color balance for clarity.

**Tumor monitoring**

At 2 months, animals with at least one area of melanocyte rescue greater than 4 mm² were selected and were scored weekly for the presence of visible tumors (Ceol et al., 2011).
CHAPTER IV

DISCUSSION

The presence of melanocyte stem cells in adult zebrafish is suggested by the unlimited capacity of melanocyte pattern re-establishment after fin amputation in the regenerating caudal fin (Rawls and Johnson, 2000). Adult zebrafish are also capable of regenerating their body stripe melanocytes from unpigmented precursors after melanocyte ablation (O'Reilly-Pol and Johnson, 2008). However, the identity of the cells that mediate melanocyte regeneration and the mechanisms that govern their activity was unknown. In this study, we took advantage of a targeted cell ablation approach to identify the cells that mediate melanocyte regeneration (Figures II.1A and II.2A and II.2B). We found that ablation of \textit{mitfa}-expressing cells causes failure of regeneration and hence the cells that facilitate melanocyte regeneration express \textit{mitfa} (Figures II.1B). Using nlsEGFP to label the \textit{mitfa}-expressing cells, we identified unpigmented progenitor cells (Figure II.3A) on the flank of adult zebrafish that get activated and differentiate after melanocyte ablation (Figure II.5). \textit{mitfa}-expressing progenitor cells give rise to adult zebrafish melanocytes during metamorphosis (Budi et al., 2011). They arise from the neural crest during development and reside in their DRG niche until they are needed (Dooley et al., 2013). It is possible that the progenitors we identified are also made during metamorphosis.
by the same mitfa-expressing cells. Alternatively, their origin could be from a different pool of cells. Either way it is likely that like melanocytes these cells or their precursors are made during development from the neural crest. One approach to identify the origin of these cells and where they remain in larvae and during metamorphosis could be to label small clones of mitfa-expressing cells in the developing neural crest, track their location in embryos, juvenile and adult zebrafish, and then assay whether they are the cells responsible for melanocyte regeneration. Alternatively, these cells could arise from a mitfa-negative precursor cell, and we could fate-map the origin of these cells by photoconverting nuclear localizing Eos (nEos) or Kaede driven mosaically by neural crest promoters in a small number of cells during development (Ando et al., 2002; McMenamin et al., 2014). In the unlikely event that these cells or their precursors do not arise from the neural crest, we could use an unbiased approach and try labeling individual cells earlier in development in the blastula or gastrula stages. These experiments would also help us know whether the same progenitor cells or their precursors exist in and also mediate melanocyte regeneration in zebrafish larvae, make new melanocytes during metamorphosis or regenerate melanocytes in the caudal fin.

It is important to note here that in our lineage-tracing experiments we could not identify the source of every single regeneration melanocyte. In order to make sure that we didn’t lose information due to cell movement or EGFP being
switched on or off in cells, we initially imaged the flanks of the zebrafish three or four times a day. We observed that we were able to track the cells by analyzing the pictures taken once a day and so we performed additional experiments by imaging every 24 hours. During analysis of the lineage tracing experiments we lost track of some cells whose fluorescence was blocked by the melanin pigment from regenerating melanocytes. We only included those cells in our analyses, which we could trace from regenerated melanocyte back to unpigmented *mitfa*-expressing cell before neocuproine ablation. In some instances, unpigmented *mitfa*-expressing cells appeared de novo, and it is possible that similar to the DRG-associated cells that give rise to metamorphosis melanocytes (Budi et al., 2011; Dooley et al., 2013), there are *mitfa*-expressing cells deep inside the fish that cannot be seen or imaged before melanocyte ablation and we are only seeing part of the picture. One way to answer this would be to make cryosections and then use in situ hybridization for the *mitfa* transcript or antibody staining for the Mitfa protein, and look for *mitfa* expression in extrahypodermal regions, especially associated with the peripheral nerves.

Alternatively, there could be a stem cell population that is *mitfa*-negative, but this would not explain why melanocytes fail to regenerate when we ablate all *mitfa*-expressing cells (Figures II.1A and II.2A). It is also possible that *mitfa*-negative stem cells must go through a *mitfa*-positive state before differentiating and in our NTR-Mtz ablation experiments we keep killing progenitors and
exhaust the stem cells. This seems unlikely, as we have shown that adult zebrafish melanocytes have a massive regeneration potential, and are capable of making new melanocytes after multiple rounds of ablation and recovery (Figure II.7A). We could address this by reducing the time fish are treated with Mtz in our ablation experiments. This would potentially only ablate the mitfa-expressing progenitor cells, but would not allow sufficient time for the mitfa-negative stem cells to keep making new mitfa-expressing progenitor cells and get exhausted.

Another interesting finding was that p53 is not required for melanocyte death but is needed for progenitor cell death. We have shown that unpigmented mitfa-expressing cells are spared on ablation in p53-deficient fish (Figure II.3D), but studies have shown that functional p53 is not required for NTR-mediated ablation and cell death proceeds through a p53-independent apoptotic pathway (Cui et al., 1999). It is possible that different cell types use a different cell death pathway when challenged with NTR-Mtz-mediated damage. Another possibility could be the expression levels of NTR in progenitor cells and differentiated melanocytes; progenitor cells express lower levels of mitfa and hence NTR which is under the control of the mitfa promoter. NTR levels could be even lower in the p53 loss of function progenitor cells or alternatively under low levels of NTR expression the cells may die in a p53-dependent way. This could be resolved by expressing NTR in these cells using a stronger promoter like ubi:switchNTR coupled with mitfa:Cre (Mosimann et al., 2011).
Experiments in zebrafish larvae using temperature sensitive *mitfa* loss of function alleles have suggested that *mitfa* is not required to establish or maintain the melanocyte stem cell that mediates regeneration in larvae or makes new melanocytes during metamorphosis (Johnson et al., 2011). Our experiments have shown that the cells that mediate regeneration in adult zebrafish express *mitfa*, but we do not yet know whether *mitfa* expression is required for their generation. A possible strategy to test this would be to similarly use the temperature sensitive *mitfa* loss of function mutant fish and perform reciprocal growth, and ablation and regeneration experiments at restrictive and permissive temperatures.

One way to understand the source of the de novo cells might be to use an unbiased approach instead of only analyzing the unpigmented *mitfa*-expressing cells during regeneration. Similar to the studies used to identify the mouse MSC (Nishimura et al., 2002), we could take advantage of label retention by slow cycling stem cells. A cell that incorporates BrdU after melanocyte ablation, but retains the label after many rounds of regeneration is likely the MSC. Another approach could be to use the fluorescent ubiquitylation-based cell cycle indicator (FUCCI) system and look for any cells that get activated and enter the S/G2/M phase after melanocyte ablation. Both techniques would have to be followed by cell ablation and lineage-tracing to confirm that these are melanocyte stem/progenitor cells, and would require identification of a marker that is specific
to that cell population. This highlights the advantage of the targeted cell ablation assay that we used, and suggests that we could use the same approach to identify other MSC/progenitor cell markers. In our current study we used promoters of genes that are known to be important to make melanocytes from neural crest cells during development, rationalizing that these genes may also be expressed in the cells that mediate melanocyte regeneration. We could expand the pool of promoters tested to include more neural crest genes, genes that are important in other stem cell systems, and those that are able to positively or negatively regulate important melanocyte genes like mitfa.

During melanocyte regeneration, we identified a prominent role for direct differentiation of progenitor cells (Figure II.5 and Table II.1). In well characterized regeneration models, such as the hematopoietic system, progenitor cells are known to differentiate without dividing, however these progenitors represent a pool of cells that are continuously turned over and are replaced by other progenitors as they differentiate (Orkin and Zon, 2008). The mitfa-expressing progenitor cells that we have identified to be responsible for melanocyte regeneration do not generally undergo proliferation and differentiation. In contrast, they are a pool of quiescent cells poised to divide or differentiate only upon injury (Table II.1). Turnover is low in some stem cell-differentiated cell systems, like the skeletal muscle, brain and heart (Beltrami et al., 2003; Collins et al., 2005; Doetsch et al., 1999; Gros et al., 2005; Zhang et al., 2008), and it is
possible that even though we have not observed significant turnover of melanocytes, our progenitor cells differentiate and replenish melanocytes at a slow rate. To test this we could either trace melanocytes or unpigmented progenitor cells on the flanks of adult zebrafish for longer periods of time in the absence of melanocyte ablation. Alternatively, we could also specifically label progenitor cells and see if the label is observed in melanocytes after an extended length of time. Similarly, we could label individual or small numbers of melanocytes and see whether they persist or disappear over long intervals of time.

It is possible that the progenitor cells are maintained in a quiescent state by a signal from differentiated melanocytes. Death of differentiated melanocytes may result in the disappearance of this putative inhibitory signal, allowing the progenitor cells to exit their quiescent state and undergo differentiation into new melanocytes to replace the cells that are lost (Bruns et al., 2014; Hsu et al., 2014; Sato et al., 2011). Alternatively, a recent study has shown that in airway epithelia, stem cells relay a signal to their daughter cells thus maintaining them; the progenitor cells differentiate in the absence of this signal (Pardo-Saganta et al., 2015). Also, the stem or progenitor cells could also regulate themselves (Lim et al., 2013). One could explore this by supplementing ligands or blocking signals in the presence or absence of melanocyte ablation.
This is reminiscent of melanocyte development where Pax3 functions to initiate a melanocyte program by activation of Mitf, while simultaneously acting downstream to prevent terminal differentiation, leaving melanoblasts committed and poised to differentiate (Lang et al., 2005). This is also similar to skin regeneration in mice, where after injury due to wounding or UV irradiation, MSCs migrate out of their hair follicle niche without dividing and make new epidermal melanocytes (Chou et al., 2013). This leads to exhaustion of MSCs in the hair follicle and hair graying in following hair cycles. Loss of epithelial tissue is potentially dangerous and to prevent this the skin had developed a rapid response where skin healing and repigmentation are given priority over stem cell maintenance in the hair follicle. Most interestingly, clinical observations have implicated hair follicle MSCs in skin repigmentation after vitiligo, a disease caused by the loss of epidermal melanocytes. Recovery is characterized by radial repigmentation of the skin surrounding hair follicles (Cui et al., 1991). It would be interesting in our model system to probe the interactions between the different stem cell progenitor cell systems that share the epidermis.

On a related note, it would also be interesting to observe whether melanocytes regenerate by direct differentiation when other forms of injury are incurred rather than specific ablation of melanocytes by neocuproine. Targeted ablation of melanocytes in zebrafish has the advantage of killing only cell type and eliciting a specific regeneration response. But zebrafish in the wild or in
captivity, most likely sustain injuries, which damage more than one cell type. It is possible that the fish responds to different injuries with a different mode of regeneration. In a setting where injury takes away a large number of cells, and only a few progenitor cells are spared, the remnant cells may resort to division in order to regenerate (Tu and Johnson, 2010). Alternatively, similar to mammalian skin after injury, repigmentation may be given priority over stem cell maintenance and more progenitor cells may directly differentiate (Chou et al., 2013). We could explore this by using different injury models on the flanks of adult zebrafish.

In our model, we discovered a prominent role for direct differentiation because we could trace single cells over time in a live animal. This may be a mode of regeneration in other systems as well, but limitations in the methods available to lineage trace cells may not provide enough resolution. A very recent study looking at regeneration of airway epithelial cells suggests that direct differentiation may be involved in that setting as well, as very few differentiated cells incorporate BrdU during regeneration (Pardo-Saganta et al., 2015).

As discussed in Chapter II, direct differentiation may enable rapid regeneration of melanocytes, which are important for predator avoidance and fish-to-fish recognition (Engeszer et al., 2004; Engeszer et al., 2008; Price et al., 2008). However, regeneration lacking any cell division, would likely result in impaired reconstitution of pigment pattern as the pool of progenitor cells is
exhausted. Coupling direct differentiation and division of other progenitor cells (Figures II.5, II.9A and II.9B), results in an extensive and perhaps unlimited capacity to regenerate melanocytes. Similarly, the interfollicular epidermis (IFE) in mice is known to regenerate epithelial cells from both stem and progenitor pools. In wound repair, the progenitor pool residing in the IFE is heavily involved initially, while descendants of the stem cell pool predominate in later stages (Mascre et al., 2012).

We also observed that during regeneration the progenitor cells initially express low levels of *mitfa* and then upregulate *mitfa* expression as they differentiate. This is consistent with the rheostat model in melanoma cells where low levels of MITF expression leads to stem cell like quiescence, increased MITF expression leads to cell division, and even higher levels of MITF expression leads to differentiation (Carreira et al., 2006).

In our lineage-tracing analyses, we observed that unpigmented *mitfa*-expressing cells either directly differentiate with no division, divide symmetrically or divide asymmetrically (Figures Figure II.5, II.9A and II.9B). Interestingly, studies using transposon-mediated cell labeling also suggest that in the regenerating fin, MSCs may divide both asymmetrically to generate new melanocytes, and symmetrically to expand MSCs and generate quiescent cells. (Tu and Johnson, 2010). It is intriguing how the progenitor cells choose what
fates to adopt during melanocyte regeneration. However, it is also possible that the different modes of division are not a decision that progenitor cells make, but that different types of progenitors are predetermined to undergo specific fates. In other stem cell-differentiated cell systems, it is debated whether a hierarchical or stochastic model is used to determine stem cell fate. Coupling \textit{mitfa}-positivity with other progenitor cell and differentiation markers like \textit{dct} or \textit{tyr} would make our model of zebrafish melanocyte regeneration amenable to answer those questions by longitudinal single cell lineage-tracing during regeneration in a live animal. It is also possible that we are not seeing the whole picture from stem cell to differentiated cell, but just part of it from progenitor cell to differentiated melanocyte. As discussed earlier, since we know that the cells that mediate melanocyte regeneration express \textit{mitfa}, one way to answer this would be to look for \textit{mitfa}-expressing cells in extrahypodermal regions, especially associated with the peripheral nerves. It would be challenging to perform single cell lineage tracing on cells located inside the fish, but we could try to do so using transparent casper fish (White et al., 2008). We could also use confocal microscopy coupled with cell labeling at timepoints before and during regeneration.

In the regenerating caudal fin, melanocyte precursor cells are uniformly distributed in the stripe, not just close to melanocytes, and are locally recruited by the amputation plane (Rawls and Johnson, 2000). We also observed unpigmented \textit{mitfa}-expressing cells uniformly distributed in the stripes and in the
interstripes as well. During development or regeneration of the caudal fin, melanocytes and xanthophores develop from common precursors, whereas iridophores come from a different lineage (Tu and Johnson, 2010, 2011). In contrast, during metamorphosis the unpigmented mitfa-expressing cells associated with the DRG give rise to melanocytes and iridophores, whereas xanthophores arise from a different source (Dooley et al., 2013). It is not known whether our precursor cells can give rise to xanthophores and iridophores during regeneration or whether they are lineage restricted melanocyte progenitors. This could be understood by ablating only xanthophores or iridophores, by using specific promoters to drive the expression of NTR, and assaying the regeneration response. Mosaic cells containing more than one type of pigment have been observed in developing fish and it is proposed that the various pigment cells are derived from a stem cell that contains a primordial organelle which can differentiate into any of the known pigmentary organelles (Bagnara et al., 1979).

In contrast to the distinct horizontal stripes of zebrafish, other species of Danio fish have different patterns including spots, stripes with breaks and irregularities, broad diffuse stripes, vertical bars, even distribution of melanocytes with no stripes, etc. (Quigley et al., 2004). In these animals it would be interesting to explore the number and arrangement of progenitor cells, and to define how progenitor cells in these fish sense and respond to injury.
Another obvious question is that of the regeneration capability of older fish. Zebrafish are able to regenerate their melanocytes after multiple rounds of ablation and regeneration (Figure II.7A), and since melanocyte turnover in zebrafish in the absence of injury appears to be low (Table II.1), it seems likely that older zebrafish would have no problem regenerating their melanocytes as well as younger fish. Alternatively, fish may lose MSCs as they age due to poor stem cell maintenance over time (Nishimura et al., 2005). We could test this by challenging older fish to regenerate their melanocytes and also by labeling small numbers of progenitor cells in younger fish and assaying their presence in older fish as progenitors or differentiated melanocytes.

Identifying signals that govern melanocyte regeneration is also critical, so we looked at pathways important in melanocyte ontogeny. In zebrafish, studies have shown that during development Wnt/β-catenin signaling promotes pigment-cell formation from neural crest cells by activating mitfa (Dorsky et al., 1998; Dorsky et al., 2002). Using transcription based reporters we observed that Wnt/β-catenin signaling is active in differentiated melanocytes but not in the unpigmented mitfa-expressing cells that mediate melanocyte regeneration (Figure II.10A). We also observed that the Wnt reporter signal is lost when differentiated melanocytes are ablated (Figure II.11), and then gets turned on in differentiating melanocytes during regeneration (Figure II.10B), and gets further upregulated as the cell differentiates. Absence of signal when the TOPFlash
reporter is replaced with the FOPFlash reporter provides evidence that the signal is specific to activated Wnt/β-catenin signaling (Figure II.10A). Further evidence is provided by preliminary antibody staining data showing the presence of nuclear β-catenin in differentiated melanocytes.

We inhibited Wnt/β-catenin signaling using IWR-1, which does so by inhibiting tankyrases and hence stabilizing the Axin destruction complex, which then degrades more β-catenin. IWR-1 has successfully been used to inhibit Wnt/β-catenin signaling in adult zebrafish and we can show that it works in the melanocyte lineage by a decrease in the TOPFlash signal in differentiated melanocytes when fish are treated with IWR-1 (Figure II.12A) (Chen et al., 2009; Huang et al., 2009; Lu et al., 2009). We observed that inhibition of Wnt signaling during melanocyte regeneration leads to fewer numbers of regenerated melanocytes (Figures II.13B and II.13C).

Since all drugs may have some non-specific activity and IWR-1 treatment acts on the entire fish, we also tried to inhibit Wnt/β-catenin signaling by expressing the Wnt inhibitor dkk1bGFP under the control of the heatshock promoter. We did not see a striking melanocyte regeneration defect when we heatshocked the fish once a day for one hour. Heatshock for a longer duration or more often lead to death of the fish. We also inhibited Wnt/β-catenin signaling genetically and specifically in melanocytes by expressing a dominant negative
version of TCF (tcfΔCGFP) under the *mitfa* promoter. Unfortunately, this allowed very few transgenic melanocytes to develop in our chimeric fish, and the melanocytes that did develop were those that expressed very low levels of the dominant negative TCF. Making a knockout of β-catenin would be the most elegant way to test whether the regeneration phenotype we see is due to the effect of inhibiting canonical Wnt/β-catenin signaling.

Regeneration can be facultative (activated by injury) or homeostatic (replacement of cells lost through wear). Signals that are important in facultative regeneration have also been shown to be important in tissue maintenance; Fgfs in zebrafish fin maintenance and regeneration are a striking example. When Fgf signaling, which plays a critical role in fin regeneration, is inhibited using heatshock inducible dominant-negative Fgfr in transgenic fish, there is a striking loss of fin tissue within 30 days. When Fgf signaling is restored, the fin structures are able to recover in 30 days (Wills et al., 2008). A question that arises is whether Wnt/β-catenin signaling is similarly needed to maintain melanocytes. We inhibited Wnt/β-catenin signaling in the absence of melanocyte ablation and did not see a decrease in the number of melanocytes (Figure II.12B and C). This suggests that Wnt/β-catenin signaling may not play a role in the homeostatic maintenance of differentiated melanocytes. We could confirm this by inhibition for a longer duration of time. Also, inhibition with IWR-1 may not be complete and
low levels of Wnt/β-catenin signaling may be adequate for tissue maintenance. We could also try using heatshock inducible \textit{dkk1b} or dominant negative \textit{tcf}.

Studies in mice have shown that Wnt activation in MSCs drives their differentiation into pigment-producing melanocytes, and that forced activation of Wnt signaling leads to premature differentiation of MSCs and their exhaustion (Nishimura et al., 2005; Rabbani et al., 2011). Concurrently, Wnt activation in the HFSCs, which share the MSC niche, promotes hair follicle formation and MSC proliferation through endothelins (Rabbani et al., 2011). In our system it would be interesting to determine the source of Wnt ligands and whether the signal is cell autonomous or not. This could be understood by making cryosections and performing in situ hybridization experiments to identify the source of the Wnt ligands. It would also be interesting to test whether expression of Wnt signaling causes exhaustion of the regenerative capacity of the progenitor cells due to forced differentiation.

Important questions that remain include what signals keep MSCs quiescent, activate them when needed, and then return them to quiescence? On the flanks of adult zebrafish, melanocyte progenitor cells appear closely associated with differentiated melanocytes (Figure II.3A), and in some cases they appear to be in contact with each other (Figure II.3B). In addition, melanocyte progenitors enter the cell cycle (Figure II.8C), and divide as quickly as two days
post neocuproine application (Figure II.8A). As mentioned earlier it is possible that there is a signal from the differentiated cell that keeps the progenitor quiescent, and loss of this cue activates the cell. We know that even though Wnt signaling is needed during melanocyte regeneration, it is not needed to maintain melanocytes. Blocking Wnt signaling in the absence of melanocyte ablation does not seem to activate the progenitor cells to make new melanocytes (Figures II.12B and II.12C). Progenitor cells may also be able to regulate themselves. We could use other transcription-based reporters to assay additional pathways. Alternatively, signals from the niche or MSC may be responsible for progenitor cell quiescence or activation, so identifying the MSC, the MSC niche and understanding the extent to which MSCs and progenitor cells are dependent on it will be useful. Signals that maintain MCSs may also play a role in halting stem cell loss, and help to prevent disease related depigmentation and premature hair graying. Similar to the hair follicle, the epithelial skin is a complex organ, and interactions between MSCs and other stem cells that may share the niche, and crosstalk between the various differentiated cell-stem cell systems that make up this tissue most likely play a role in tissue homeostasis. A recent study showed that along with local factors, global factors, like hormones, also influence pigment cell development and maintenance in adult zebrafish. Thyroid hormone (TH) limits melanophore expansion and thyroid ablation leads to more melanocytes during metamorphosis and in the adult pigment pattern (McMenamin et al., 2014). It is possible that like TH, other hormones and global factors may
influence adult stem/progenitor cells and regeneration as well, and would be interesting to explore.

Dysregulation of melanocytic growth is a common occurrence and almost all people harbor multiple benign nevi (moles), which only rarely become invasive melanomas. Some of the genes that are commonly mutated in melanoma like \( \text{BRAF}^{V600E} \) and \( \text{NRAS}^{Q61K} \), have been identified, but these alone cannot explain all aspects of melanoma initiation. Many pathways that are important in stem cell development and function are dysregulated in cancer (Reya et al., 2001). It is also hypothesized that plasticity in the hierarchical organization of a tissue may play a role in tumor initiation. Our observation that Wnt signaling is active in differentiated melanocytes, but not in the melanocyte progenitor cells, prompted us to investigate the role of Wnt signaling in melanoma initiation. We found that Wnt signaling is turned off in about half of the tumors, and inhibition of Wnt signaling in melanocytes leads to acceleration of melanoma onset (Figures III.1B and III.2)

Although it is well known that Wnt signaling is frequently dysregulated in melanoma, the functional implications of this observation are unclear. Loss of nuclear \( \beta \)-catenin is associated with melanoma progression in human tumors. One study demonstrated that in malignant melanoma, elevated levels of nuclear \( \beta \)-catenin in human primary tumors and metastases correlate with reduced
proliferation and with improved survival. Melanoma cells expressing a Wnt ligand also exhibited decreased tumor size and decreased metastasis when implanted into mice. Transcriptional profiling revealed that the Wnt ligand upregulated genes implicated in melanocyte differentiation, several of which were downregulated with melanoma progression (Chien et al., 2009). Similarly, in both intestinal and skin epithelia where Wnt signaling plays the opposite role and is active in stem cells, activation of Wnt signaling has been linked to malignancy (Chan et al., 1999; Gat et al., 1998; Morin et al., 1997).

On the other hand, by modulating β-catenin levels in a mouse model of melanoma, it has been demonstrated that β-catenin is a central mediator of melanoma metastasis to the lymph nodes and lungs, and β-catenin levels control tumor differentiation and regulate both MAPK/Erk and PI3K/Akt signaling. These findings establish Wnt/β-catenin signaling as a metastasis regulator in melanoma (Damsky et al., 2011). In a recent study, molecular analysis of human metastatic melanoma samples revealed a correlation between activation of the Wnt/β-catenin signaling pathway and absence of a T-cell gene expression signature. It was found that the melanoma-cell-intrinsic Wnt/β-catenin signaling pathway contributes to a lack of T-cell infiltration and resistance to anti-PD-L1/anti-CTLA-4 monoclonal antibody therapy in melanoma (Spranger et al., 2015). Taken together, it appears that the Wnt/β-catenin signaling is involved in multiple aspects of tumorigenesis, which will require continued exploration. The next step
should be to explore whether melanomas arise from differentiated melanocytes or from progenitor cells, what causes these cells to get transformed, and whether melanocyte progenitor cells are related to the cells that give rise to and maintain melanomas.
Future directions

Identifying a melanocyte progenitor cell specific marker

The identification of mitfa-positivity as a melanocyte progenitor cell marker has allowed us to perform lineage-tracing experiments and understand how source cells make new melanocytes during regeneration. However, mitfa is also expressed by differentiated melanocytes and another pigment cell, the xanthophore. We were able to exclude these cells in our lineage-tracing experiments by ablating the differentiated melanocytes and then not considering cells that had yellow pigment in our analyses. One approach to find a progenitor cell specific marker could be to use FACS to isolate unpigmented mitfa-expressing cells in fish with an fms loss of function mutation (Parichy et al., 2000; Parichy and Turner, 2003), which lack xanthophores. Comparison of RNA-Seq data between differentiated melanocytes and unpigmented mitfa-expressing cells could then be used to identify potential progenitor cell specific markers. The cells expressing this marker could then be assayed for their ability to make new melanocytes by the targeted cell ablation approach described in Chapter II, followed by lineage-tracing.

Analysis of Wnt/β-catenin signaling in melanocyte regeneration

We have identified that canonical Wnt/β-catenin signaling is important in melanocyte regeneration and we started to assay its role in more detail using genetic approaches and drug studies. The best way to confirm that Wnt/β-catenin
signaling is required for melanocyte regeneration, would be to make a knockout of \(\beta\)-catenin, but these animals would likely die early in development. A different strategy could be to use somatic mutagenesis to make mutations specifically in melanocytes. This could be transposon, ZFN, TALEN or CRISPR/Cas9 mediated. But, since genes that are important in development may also be the genes that are needed for regeneration, making a melanocyte specific mutation in \(\beta\)-catenin, may not allow melanocytes to be made in the first place. We could circumvent this by making inducible mutations after melanocytes have developed. This could be achieved by conditional genetic ablation using a combination of an inducible \(\text{Cre}^{\text{ERT2}}\) under the control of a melanocyte specific promoter like \textit{mitfa}, and a \(\beta\)-catenin null allele created by loxP sites flanking essential exons. It is important to note that it is difficult to make zebrafish knockins that do not disrupt the targeted endogenous gene. An alternative strategy could be to use \textit{ubi:Switch} \(\text{DN}\beta\text{-catenin}\) and \textit{mitfa: \text{Cre}^{\text{ERT2}}}, to express dominant negative \(\beta\)-catenin specifically in melanocytes after they are generated.

**Exploring the role of additional signaling pathways in progenitor cell maintenance and differentiation**

In our studies, we identified Wnt signaling as an essential pathway in progenitor cell differentiation during melanocyte regeneration. After melanocyte ablation Wnt signaling gets turned on in unpigmented \textit{mitfa}-expressing cells, and inhibiting Wnt signaling perturbs melanocyte regeneration. Wnt appears to be
important late in the regeneration process, as differentiation follows appearance of TOPFlash signal. It would be interesting to identify other signals, especially those that are involved in progenitor cell maintenance during homeostasis and in the early stages of progenitor cell activation after injury. This could be accomplished similar to the identification of Wnt by using reporter lines (Moro et al., 2013; Schiavone et al., 2014) and drugs for common pathways like Notch, Shh, BMP and TGFβ. The RNA-Seq data obtained could also help identify new pathways.

**Exploring the role of other signaling pathways important in regeneration in melanoma initiation**

Using the differential activity between melanocytes and melanocyte progenitor cells, we discovered a potential role for Wnt signaling in melanoma initiation. We could use the RNA-Seq data, followed by reporter assays, genetic tools, and drugs to explore the role of other pathways in melanoma initiation. Some of these pathways may also be important in melanoma maintenance or tumor resistance, and would be interesting to evaluate.
REFERENCES


potential enzyme for antibody-directed enzyme prodrug therapy (ADEPT). Biochemical pharmacology 44, 2289-2295.


Proceedings of the National Academy of Sciences of the United States of America 111, 5349-5354.


ligase that is essential for efficient activation of Notch signaling by Delta. Developmental cell 4, 67-82.


