Zebrafish genetics: Fish and ChIPs and beyond

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The emergence of cancer

Cancer-prone cells or “cancerized field” → Cancer Initiation → Tumor Expansion

“FIELD CANCERIZATION” IN ORAL STRATIFIED SQUAMOUS EPITHELIUM
Clinical Implications of Multicentric Origin
DANIEL P. SLAUGHTER, M.D., HARRY W. SOUTHWICK, M.D.,
AND WALTER SHEHAT, M.D. CANCER September 1953

Oncogene Activation
And/or
Tumor Suppressor Loss
Potentially oncogenic mutations in “normal” tissue

"Normal" eyelid skin

"Normal" esophagus

Marticorena et al, Science, 2015

Marticorena et al, Science, 2018

The emergence of cancer

Normal Tissue
Cancer-prone cells or “cancerized field”
Cancer Initiation
Tumor Expansion

Oncogene Activation
And/or
Tumor Suppressor Loss

Change in transcriptional or epigenetic state?

Outline

I. Why zebrafish?
II. Genetic screening in zebrafish - old school to new school
III. “Modern” genetic approaches in zebrafish
IV. Applications to neural crest development and melanoma cancer

I. Why zebrafish?

• Goal of developing a genetically tractable vertebrate model system
I. History of zebrafish as a model organism

• George Streisinger – founding father

• Trained with phage biologists (many "reformed" physicists) - dawn of molecular genetics.
• Mutational approaches in bacteria -> gene function.
• Brenner and Benzer - logic of complex systems could be deconstructed using mutation-based genetic analysis.

Why zebrafish?

• Goal of developing a genetically tractable vertebrate model system

Why zebrafish?

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Why zebrafish?

- Goal of developing a genetically tractable vertebrate model system
  - Medaka
  - Whitecloud Mountain Fish

Why zebrafish?

- Breed very well in the laboratory
  - amenable to genetic analyses
  - breed year-round
- External fertilization
  - gametes can be harvested separately
- Development is readily observable
- Isolated from Ganges River

iSpawn – large scale breeding

Isaac Adatto and Techniplast
Zebrafish development is readily observable (and fast)

- Rapid, transparent development
  - many tissues form by 24 hours
  - *in vivo* imaging

Disadvantages of zebrafish

- Obstacle: efficient recovery of mutant phenotypes in a diploid vertebrate.
- ID rare recessive mutations and propagate them in the (unaffected) heterozygous carrier.
  - *C. elegans*: single +/- carriers can produce +/- and +/+ siblings.
  - *Drosophila* had 50 years worth of genetic tricks, like marked and balancer chromosomes.
- Lack of genetic markers would make tracking affected regions of the chromosome difficult.
- Streisinger spent over a decade establishing zebrafish (husbandry/embryology) and developing tools to quickly (one generation) recover recessive mutations from the germ line.

II. Streisinger et al. 1981 – the first cloned vertebrate

First efforts focused on the maternal germ line

- Landmark 1981 paper
  - Highly efficient method for activating the development of eggs without genetic contribution from the sperm
  - Allows recovery of mutants in one generation.
- Can live ~3 days as haploid organisms
  - Rapidly ID mutations affecting embryonic development.
Haploid screens

More recently:

- gamma-ray (UV cross-links DNA)
- no genetic contribution from the male

Haploid screen - advantages

- Cheaper and faster
- Mutant recovery in one generation
- No need to raise many F2 families
- Useful for:
  - identifying changes in early development caused by mutations
  - mutations in mutagenized females
  - identifying mutation-bearing heterozygous females

Haploid screen - disadvantages

- Midblastula transition
  - Slower cell divisions and zygotic transition begins occurs one cleavage later in haploids than diploids
- Haploids
  - Smaller and more cells than diploids
  - Inviable

Emergence of a community

- Eugene, OR - zebrafish central
- Mid-70s, Chuck Kimmel begins work on the zebrafish
  - neuroanatomy
  - describes more neurons in zebrafish than had been recognized in any other vertebrate
  - fate maps
- Kimmel and Streisinger - large scale collaborative screens planned
  - early patterning and differentiation of the nervous system
1927-1984

Early screens from Eugene: $\gamma$-ray-induced mutations

- cyclops
- no tail
- spadetail

- wild type
- cyclops
- = brachyury
- = nodal

Pitfalls of $\gamma$-ray induced mutations

- Genetic alterations that arise from ionizing radiation vary
  - point mutations
  - large deletions*
  - translocations*
  * affect more than one gene
- Not ideal for saturation screens: better to have a mutagen that induces lesions in single genes.

II. Zebrafish expand: The "Big Screen"

Christiane Nüsslein-Volhard
Max Planck Institute
Tübingen

Wolfgang Driever
Massachusetts General Hospital
Boston

Recapitulate the Drosophila screen for embryonic pattern mutants in a vertebrate.
Classic three-generation scheme
Mutations induced in the parent generation are driven to homozygosity in the F3 generation.

- P: Pre-meiotic spermatogonia are mutagenized
- F1: non-mosaic heterozygotes each carrying one or more mutations.
- F2: 50% of F2 animals are +/- for the mutation inherited from the F1 founder
  - ¼ of matings have potential to be informative!
- F3: F2 siblings are crossed, and homozygous mutant phenotype is seen in 25% of progeny
  (from ¼ of matings)

“The Big Screen”
- Tübingen and Boston
  - ~4000 embryonic lethal mutant phenotypes recovered.
- Instead of “slow trickle”, 37 papers published in a single volume of Development.

Development Volume 123
A taste of the mutant phenotypes
- unique and essential functions
- embryogenesis
- epiboly
- gastrulation
- dorsoventral patterning
- notochord formation
- midline and body shape
- somite formation and patterning
- digestive organs
- jaw and brachial arches
- axon pathfinding
- retina development
- brain development
- midbrain/hindbrain boundary formation
- forebrain development
- neural survival
- neural degeneration
- inner ear and lateral line
- fin formation
- cardiovascular system
- hematopoiesis
- craniofacial development
- pigmentation
- locomotion
Going from mutant phenotype to mutation

- Identify candidate genes.
- Positionally clone the mutation.

Candidate gene approach

- Assemble cloned genes that have expected properties of the mutated locus.
- Test these genes as candidates:
  - Expression pattern
  - Mutant phenotype in other species
- Drawback
  - Very subjective
  - Easy to fall in love with the wrong gene…

Positional cloning

- Unbiased approach
- If genetically tractable, mutation can hit any biochemical pathways
- Zebrafish genome is large, but is amenable to positional cloning projects.
  - high fertility: analysis of 1000’s of meioses and fine mapping to a small interval
  - external development: test candidate genes in an interval by rescue and orthogonal loss of function approaches

Positional cloning – old method

1. Identify DNA segments (“markers”) linked to mutant locus.
   - simple sequence length polymorphisms (SSLPs)
   - >3500 primer pairs available commercially
2. Correlate markers with genomic maps
   - ID “the critical region” containing mutant locus
3. Identify the causative gene within the critical region:
   - sequence analysis
   - phenocopy with new alleles (TALENs/CRISPRs)
   - transgenic rescue of mutants with the WT gene.
With the advent of deep sequencing...

Whole Genome Sequencing to identify causative lesions

III. Other approaches in zebrafish to study gene function

Screening-based approaches:
- Insertional mutagenesis

Targeted approaches:
- TILLING
- Morpholinos
- Zinc finger nucleases
- TALENs
- CRISPR/Cas
- Transgenic strategies
Insertional mutagenesis

- Retroviral insertions
  - Pioneered by Nancy Hopkins at MIT.
- Transposon-based gene trap vectors
  - Pioneered by Koichi Kawakami at the National Institute of Genetics

Mutation induced by the insertion, and the introduced DNA sequence can be used as a tag to quickly clone the mutated gene.

Current protocols are less efficient at disrupting genes than chemical mutagenesis, but ease of isolating the disrupted gene is attractive.

TILLING

- Targeting Induced Local Lesions in Genomes
  - Cecilia Moens (Fred Hutchinson Cancer Research Center)
  - Lila Solnica-Krezel (Wash U)
  - John Postlethwait (U Oregon, Eugene).
- Library of 8,640+ ENU-mutagenized zebrafish
  - screened re-iteratively for mutations in genes of interest

Morpholinos...

- Antisense oligonucleotides
- Block translation OR splicing
- Morpholine ring instead of ribose or deoxyribose, ~25 morpholino subunits long.
- Designed to bind target RNA.
  - Translation blocking: flanks start site.
  - Splice blocking: flanks splice junctions or splice regulatory sites.
- Useful, but concern for producing off-target, nonspecific effects.
- Short acting (3-5 days of development)

...are controversial

- Poor morpholino/mutant phenocopy rates
- Morpholinos still cause defects, even in mutants where MO target site is absent.
Updated guidelines one should follow


Updated guidelines one should follow

Oct 19, 2017

Fig 1. Flowchart for zebrafish researchers interested in using morpholinos (MOs) to study the function of gene x.


Recent advances in genome editing allow rapid mutant generation

• Zinc fingers – fusion to FokI nuclease
• TALENs - Transcription Activator-Like Effector Nucleases
• CRISPR/Cas9 – has taken over…

CRISPR/Cas9 System

• Immune defense mechanism used by bacteria and archaea to protect against foreign nucleic acids (e.g. invading viruses and plasmids)
• CRISPR: Clustered Regularly Interspaced Palindromic Repeats
• Cas9: CRISPR associated protein 9

(Doudna and Charpentier, Science, 2014)
CRISPR/Cas in zebrafish

Efficient genome editing in zebrafish using a CRISPR-Cas system

CRISPRs in practice

- Numerous gRNA design tools online (e.g. CHOPCHOP)
- Synthesize gRNA and Cas9 mRNAs using standard methods
  - OR
  - Pre-formed Cas9 protein/gRNA particles may be more efficient
- Inject into single cell embryo using microinjection
- Screen animals for mutations, insertions, etc.
- Controls for off-target effects is essential
  - Non-complementation of independently derived alleles

TEAZ: Transgenic Electroporation of Adult Zebrafish

Adapted from Callahan et al., 2018

Versatility of TEAZ: skin and melanoma model

Adapted from Laxamana et al., 2018
Zebrafish in practice - chemical genetics and precision medicine

**LETTERS**

**NATURE MEDICINE**

**ARAF recurrent mutation causes central conducting lymphatic anomaly treatable with a MEK inhibitor**


Zebrafish in practice - chemical genetics and precision medicine

- Generalized lymphatic anomaly (GLA) –
  - multifocal lymphatic anomaly
  - multiple areas of micro/macrocystic lymphatic malformation
  - often involves bone destruction

- Phenocopy lymphatic overgrowth in zebrafish embryos
  - Transgenic ARAF S214P expression in lymphatics
  - Correct the phenotype with MEK inhibitor drug cobimetinib (also used to treat melanoma)
- Treatment with cobimetinib does not cause defects during larval development
- Based on this, got FDA approval to treat child

Fluid retention fixed with cobimetinib!

IV. Applications to neural crest development and melanoma cancer

The emergence of cancer

Normal Tissue → Cancer-prone cells or “cancerized field” → Cancer Initiation → Tumor Expansion

- Oncogene Activation
- And/or Tumor Suppressor Loss
The emergence of melanoma - an epigenetic phenomenon?

Melanocyte → Nevus/Mole → Melanoma

- BRAF V600E
- Reminiscent of normal development
- Potentially modifiable

BRAF V600E → p53 loss → TERT increase → others

Melanoma Skin Cancer

- Significant cause of cancer death
  - ~10,000 deaths in US in 2018 (SEER data)

- Increasing incidence
  - Rate increasing 1.4% per year

- Alarming attributes
  - Historically, very poor prognosis in metastatic disease

Fig. 1. Clinical images of melanomas. Subtypes of melanoma include superficial spreading melanoma (A), acral lentiginous melanoma (B), nodular melanoma (C), acral lentiginous melanoma (D), and uveal melanoma (E). Images courtesy of H. Tsao, C.H. Woei, and I. Kim.
Why wear sunscreen?

Lo and Fisher, Science, 2014

The prevalence of somatic mutations across human cancer types.

Melanoma Skin Cancer

- Recurrent driver mutations (cutaneous)
  - \textit{BRAF}, RAS, \textit{NF1}, \textit{other}

Immunogenic
  - lymphocytic infiltrate

Cancer Genome Atlas Network, Cell, 2015
Melanoma Skin Cancer

- Remarkable recent advances
  - Kinase targeted ("-ib" drugs)
    - BRAF or MEK
  - Immunomodulatory therapies ("-ab" drugs)
    - CTLA-4 or PD-1

- Ipi + Nivo (2015)
- cobimetinib + vemurafenib (2015)
- Encorafenib + Binimetinib (2018)
- Adapted, Lo and Fisher, Science, 2014

- TVEC - oncolytic virus (2015)

- Lo and Fisher, Science, 2014

Zebrafish melanoma model

- Tg(mitf:human BRAF(V600E)), p53 lof

- NRAS, HRAS, and NF-1-driven cutaneous models
- GNAQ/11-driven uveal melanoma model
- SPRED1 mucosal melanoma model

The emergence of melanoma - an epigenetic phenomenon?

A. Transcriptional and epigenetic events during melanoma initiation
SoxE family of transcription factors

B. Metabolic modifiers of melanoma onset
Caloric intake, fasting, and cancer initiation

C. Recurrent, non-coding mutations in putative enhancers in human melanoma
Human genomics and the zebrafish model

How to monitor melanoma initiation in vivo?

The crestin gene:
  i) zebrafish embryonic neural crest marker

Neural Crest

How to monitor melanoma initiation \textit{in vivo}?

The \textit{crestin} gene: i) zebrafish embryonic neural crest marker

The emergence of melanoma

\textbf{\textit{crestin:EGFP} as the tumor sensor?}

\begin{itemize}
  \item \textit{BRAF}\textsubscript{V600E} and \textit{p53} loss
\end{itemize}

The \textit{crestin} gene

\begin{itemize}
  \item Zebrafish multicopy retroelement with no known function
\end{itemize}
The *crestin* gene

- Zebrafish multicopy retroelement with no known function

The Tol2kit: A Multisite Gateway-Based Construction Kit for Tol2 Transposon Transgenesis Constructs

Kristen M. Kwan,¹,² Esther Fujimoto,¹ Clemens Grubher,² Benjamin D. Mangum,² Melissa E. Hardy,² Douglas S. Campbell,¹ John M. Parratt,¹ H. Joseph Yost,² John P. Ranki,¹ and Chi-Hin Chien ¹,²

The *crestin* gene

- Zebrafish multicopy retroelement with no known function

**Tol2 Kit**

- Inject plasmid DNA with “Tol2 arms” (recognition sequence) + Tol2 mRNA (to produce transposase)
- Random, high efficiency integration
- >30% germ line transmission
**crestin** - neural crest marker

**crestin:EGFP** - migration of neural crest during embryogenesis

- Genetic lineage trace to melanocytes, jaw cartilage, Schwann cells

**crestin:EGFP** - a highly specific melanoma tumor sensor
crestin:EGFP - longitudinal tracking of melanoma tumor formation

- patches precede and are predictive of melanoma appearance
  - e.g. 30 of 30 tracked patches became tumors
- all tumors in p53/BRAF are EGFP (+)

crestin:EGFP - visualizing melanoma initiation at a single cell level in vivo

- earliest visualization of in vivo melanoma initiation

Barriers to melanoma initiation

Conclusions:
Reemergence of neural crest identity occurs during melanoma initiation

Favor neural crest metabolism formation
Overexpress sox10 → Faster onset
Inhibit neural crest metabolism formation
Melanocyte-specific inactivation of sox10 → slower onset
The emergence of melanoma - an epigenetic phenomenon?

A. Transcriptional and epigenetic events during melanoma initiation

Understanding the regulation of the transcriptional programs that change during melanoma initiation will lead us to the “triggers” of cancer onset.

1) How is sox10 regulated?
2) What other factors are involved?

Labeled melanocytes and melanoma cells enable sorting

RNA-Seq:
Confirmed upregulation of neural crest genes in melanoma vs melanocyte

crestin
sox10
sox5/6
tfap2a/e
pax3a
dlx1a/2a
nmyc
rxr
zeb1b/zeb1a
phf12b
wnt7b
tcf711
mef2cb

Martik and Bronner, Trends in Genetics 2017
Zebrafish melanoma cells
Zebrafish melanoma cell lines
Adult zebrafish melanocyte

ATAC-Seq:
- consistent open chromatin patterns across multiple tumors
- regions “open” near sox10 in melanoma vs melanocytes

sox10 regulates melanoma initiation – how is it regulated?

Inject putative enhancer construct → Screen for NC activity at 24 hpf → Generate stable lines → Screen for melanoma activity in tumors

9 out of 11 open chromatin regions can drive EGFP reporter expression in neural crest cells

A case study: Peak5 enhancer activity
- Huge upregulation of EGFP reporter in melanomas
- In multiple independent stable lines

Random transgene integration, genetic mosaics, founders (F0’s)
A case study: Peak5 enhancer activity

- Upregulation of EGFP reporter in early (pre-clinical) melanomas

Using sequence conservation to guide analysis: Peak5

What transcription factors regulate Peak5 in neural crest and melanoma?

- necessary for full neural crest-specific activity
- sufficient for neural crest-specific activity
- TUMORS?
Using sequence conservation to guide analysis: Peak5

Peak5 conserved sequence:

- necessary for full neural crest-specific activity
- sufficient for neural crest-specific activity
- TUMORS -> peak5:Δ192 loss of GFP

Hypothesis: SoxE (e.g. Sox10) binding sites crucial for neural crest (and melanoma) activity of Peak5

Hypothesis: SoxE (e.g. Sox10) binding sites crucial for neural crest (and melanoma) activity of Peak5

Intact SoxE binding sites are necessary for neural crest-specific enhancer activity of Peak5

How do individual open chromatin regions function in their native context at sox10?

- Is neural crest- and/or melanoma-specific expression of sox10 more dependent on certain enhancers/TF’s?
- Is there redundancy in the system? Have we uncovered “false” enhancers?

*CRISPR-mediated deletion of region of interest*
Deletion of sox10 minimal promoter – loss of embryonic sox10 expression

Deletion of sox10 minimal promoter – loss of melanocytes in embryos - phenocopy of sox10 +/- (colourless)

Stable Line
sox10min: 200 bp deletion
- Not viable beyond ~10 days

Deletion of peak5 – diminished sox10 embryonic expression

Deletion of peak5 – disrupted adult melanocyte pigment pattern!

Tumor onset rate?
Deletion of sox10min and peak5 – increased disruption of melanocyte pigment pattern

- Trans-heterozygous phenotype with peak5 and sox10min alleles
- Genetic interaction between alleles
- Sensitized background for sox10 interactors?

Model: Peak5 is a critical regulator of sox10 activity in neural crest and melanocytes via autoregulation by Sox10

- Likely multiple inputs
- What effect does Peak5 deletion have on melanoma formation? - pending
- The future - dCas9-inhibitor and activator chromatin modifier fusions

What other factors are involved?

- Critical gene(s) and CRE(s)
- Differential gene expression (RNA Seq)
- Differential peak analysis (ATAC-seq)
- Integration with human melanoma microarrays (16 genes)
Candidate genes identified by combining zebrafish and human data sets

- sox9b
- sox4a
- etv5b
- etv4
- dlx2a
- pax3a
- sox10
- nr2f5
- mitfa
- nr2f2
- atoh8
- alx4b
- foxc1b
- foxd1
- runx2b
- foxc1a

Differentially expressed in melanoma, differentially accessible chromatin nearby, neural crest transcription factors

Does sox9b overexpression alter the rate of melanoma onset?

- Overexpress candidate gene in melanocytes (mitf promoter) and assay rate of melanoma onset

sox9b overexpression in melanocytes slows melanoma onset

sox9b inactivation in melanocytes - increases melanoma onset rate!
Model for sox9b action

Model for sox9b action

Melanoma initiation

The emergence of melanoma - an epigenetic phenomenon?

A. Transcriptional and epigenetic events during melanoma initiation
   1. Sox10/Sox9 -
      • Differential binding? Relative levels? Homo/heterodimers?
   2. Sox10 complexes/cofactors
   3. Cross-species analysis
      - Human and zebrafish enhancers – focus on SoxE dimer sites

A. Transcriptional and epigenetic events during melanoma initiation
   SoxE family of transcription factors

B. Metabolic modifiers of melanoma onset
   Caloric intake, fasting, and cancer initiation

C. Recurrent, non-coding mutations in putative enhancers in human melanoma
   Human genomics and the zebrafish model
B) Metabolic modifiers of melanoma onset

- Caloric restriction leads to longer life
  - “La vita sordita” = “the sober life” - Luigi Cornaro b.1464
- Worms, fruit flies, rodents, primates
- Improved metabolic parameters (e.g. diabetes) and less cancer

Does altered feeding affect tumor onset?

Feed
4X daily
2X daily
1X daily

Increased feeding speeds tumor formation

What about melanoma initiation?

<table>
<thead>
<tr>
<th>Length S (cm)</th>
<th>Feeding Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.0 ± 0.17</td>
<td>4X</td>
</tr>
<tr>
<td>3.0 ± 0.12</td>
<td>2X</td>
</tr>
<tr>
<td>2.6 ± 0.19</td>
<td>1X</td>
</tr>
</tbody>
</table>

Vadim Grigura, Barbier, Zarov, and Kaufman, Biol Open, 2018
Feeding amount alters melanoma initiation

Intermittent fasting – protective against melanoma initiation

Intermittent fasting – protective against persistence of early patches?

Intermittent fasting – protective against persistence of early patches?
Integrating metabolomic analysis and genetics/epigenetics

1. What happens to these early melanoma tumors - Apoptosis? Autophagy?
2. What happens when "refeed" fish after tumor regression?
3. What metabolic and endocrine pathway(s) mediates the effect?
   A. Can we use drugs to achieve the same effect?

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Integrating metabolomic analysis and genetics/epigenetics

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Integrating metabolomic analysis and genetics/epigenetics

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Integrating metabolomic analysis and genetics/epigenetics

1. What happens to these early melanoma tumors –
   Higher resolution imaging of "fasted" tumor cells
   - Apoptosis? – cleaved caspase 3 staining
   - Autophagy? – complicated literature in melanoma

2. What happens when "refeed" fish after tumor regression?
   Complete regression - "cured"?
   - Partial regression – rapid rebound growth 😊

3. What metabolic and endocrine pathway(s) mediates the effect?

C. Recurrent, non-coding mutations in human melanoma

Human genomics and the zebrafish model

Melanoma is a highly mutated cancer

C) Recurrent non-coding variants in human melanoma

I) Identify putative cis-acting somatic variants in melanoma
II) Determine whether recurrently mutated regulatory regions affect gene expression
III) Characterize effect of candidate target genes on melanoma onset and progression

Paula Godoy
Genome-wide identification and prioritization of putatively functional non-coding somatic variants in melanoma

183 melanoma samples
- 20,894,255 substitutions
- 96,487 small insertions/deletions (indels)
- 3,868,606 hotspots inside MRRs
- 214 hotspots with FDR-adjusted p-values = 0
- 3,605 mutations

Using Massively Parallel Reporter Assays to validate cis-regulatory effect of variants

MPRA will uncover functional variants in a variety of cell states
CDC20 – promoter variants lead to decreased expression in human melanoma cell lines in MPRA (and luciferase reporter assays)

MPRA Results for G529A

Comparison of CDC20 expression across different cell lines

Effect of CDC20 expression level on SKCM patient survival

Log Fold-Change

HEK 293FT A375 SK-MEL-5 UACC-62

Expression Level

High expression (n=116) Low/Medium expression (n=343)

CDC20 “hotspot” – small CRISPR-mediated deletion leads to decreased expression, decreased motility in scratch assay

CDC20 “hotspot” CRISPR results

Conclusions and Ongoing approaches

• Reemergence of neural crest progenitor identity important during melanoma initiation

• Profiling of transcriptional and epigenetic changes during transition from melanocyte to melanoma

• Metabolic perturbations modify melanoma initiation/survival of early melanoma tumor cells

• Identifying and functionally characterizing non-coding variants
My lab and clinic

Current lab members
Eva Kramer
Jonathan Spalding
Paula Godoy
Sophia DeGeorgia
Catie Newsom-Stewart
Alex Jabari Vivier
Brennan Lord-Howe
Amy White

Current/recent rotations
Megan Glaeser
Michael Hilzendeger
Catie Newsom-Stewart

Former lab members
Rebecca Cunningham, PhD
Anna Zarov
Cade McMenus
Shayana Seneviratne
Vadim Grigura
Megan Barbier

Clinic – Tuesday AM – South County Siteman
- Melanoma, MCC, BCC, SCC

We put the BRAF in zeBRAFish!