Zebrafish genetics: Fish and ChIPs and beyond

February 15, 2018

ckkaufman@wustl.edu

https://kaufmanlab.wustl.edu/
Neural Crest

• The “fourth” germ layer
• Arises during gastrulation
  – at ~5.25 hours of age in zebrafish
• Migratory
  – Epithelial to Mesenchymal Transition

Neural Crest

• Diverse progeny

Neural Crest

- What factors drive formation of the neural crest and its descendant lineages?
Cancer = not good

• How do we use this developmentally and genetically tractable organism to better understand cancer biology?
“FIELD CANCERIZATION” IN ORAL STRATIFIED SQUAMOUS EPITHELIUM

Clinical Implications of Multicentric Origin

DANELY P. SLAUGHTER, M.D., HARRY W. SOUTHWICK, M.D.,
AND WALTER SMEJKAL, M.D.

Cancer September 1953
- Multiple separate cancers in the same patient in the same tissue
“FIELD CANCERIZATION” IN ORAL STRATIFIED SQUAMOUS EPITHELIUM

Clinical Implications of Multicentric Origin

Danely P. Slaughter, M.D., Harry W. Southwick, M.D., and Walter Smejkal, M.D.

Cancer September 1953

From the foregoing observations it would appear that epidermoid carcinoma of the oral stratified squamous epithelium originates by a process of “field cancerization,” in which an area of epithelium has been preconditioned by an as-yet-unknown carcinogenic agent.
The emergence of cancer

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

Shared exposure

Oncogene Activation and/or Tumor Suppressor Loss
The emergence of neural crest

Neuro-ectoderm → Neural plate border → Neural crest formation → Expansion and migration

*Shared exposure (morphogens?)*
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
Streisinger’s history

- 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.
- Moved to the University of Oregon, Eugene, in 1960.
Why zebrafish?

• Goal of developing a genetically tractable vertebrate model system

Medaka
Why zebrafish?

• Goal of developing a genetically tractable vertebrate model system
Why zebrafish?

• Goal of developing a genetically tractable vertebrate model system
Why zebrafish?

- Goal of developing a genetically tractable vertebrate model system
Why zebrafish?

- Goal of developing a genetically tractable vertebrate model system

Medaka  Whitecloud Mountain Fish  Zebrafish
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
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

Swinburne et al, PLOS One, 2015
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:

(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 F$_2$ 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

Kroeger et al, JOVE, 2014
## Haploid screen - disadvantages

<table>
<thead>
<tr>
<th>Normal diploid</th>
<th>Normal diploid = d</th>
<th>Normal haploid = A</th>
<th>Abnormal haploid = B, C</th>
</tr>
</thead>
</table>

- 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

Kroeger et al, JOVE, 2014
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**

<table>
<thead>
<tr>
<th>cyclops</th>
<th>no tail</th>
<th>spadetail</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>$= brachyury$</td>
<td>$= aT$-box transcription factor</td>
</tr>
</tbody>
</table>

= 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 beyond Eugene: 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.
Choice of mutagen: ENU

Efficient Recovery of ENU-Induced Mutations From the Zebrafish Germline

Lilianna Solnica-Krezel, Alexander F. Schier and Wolfgang Driever

Cardiovascular Research Center, Massachusetts General Hospital and Harvard Medical School, Charlestown, Massachusetts 02129

Manuscript received August 27, 1993
Accepted for publication December 29, 1993

ABSTRACT

We studied the efficiency with which two chemical mutagens, ethyl methanesulfonate (EMS) and N-ethyl-N-nitrosourea (ENU) can induce mutations at different stages of spermatogenesis in zebrafish (Brachydanio rerio). Both EMS and ENU induced mutations at high rates in post-meiotic germ cells, as indicated by the incidence of F2 progeny mosaic for the alt1ene mutation. For pre-meiotic germ cells, however, only ENU was found to be an effective mutagen, as indicated by the frequencies of non-mosaic mutant progeny at four different pigmentation loci. Several mutagenic regimens that varied in either the number of treatments or the concentration of ENU were studied to achieve an optimal ratio between the mutagenicity and toxicity. For the two most mutagenic regimens: 4 × 1 hr in 3 mM ENU and 6 × 1 hr in 5 mM ENU, the minimum estimate of frequencies of independent mutations per locus per gamete was 0.9–1.3 × 10−5. We demonstrate that embryonic lethal mutations induced with ENU were transmitted to offspring and that they could be recovered in an F2 screen. An average frequency of specific-locus mutations of 1.1 × 10−7 corresponded to approximately 1.7 embryonic lethal mutations per single mutagenized genome. The high rates of mutations achievable with ENU allow for rapid identification of large numbers of genes involved in a variety of aspects of zebrafish development.

Genetics 136: 1401–1420 (April, 1994)

Large-scale mutagenesis in the zebrafish: in search of genes controlling development in a vertebrate

Mary C. Mullins, Matthias Hammerschmidt, Pascal Haffter and Christiane Nüsslein-Volhard

Max-Planck-Institut für Entwicklungsbiologie, Spemannstrasse 35/18, 72076 Tübingen, Germany.

Background: In Drosophila melanogaster and Caenorhabditis elegans, the elucidation of developmental mechanisms has relied primarily on the systematic induction and isolation of mutations in genes with specific functions in development. Such an approach has not yet been possible in a vertebrate species, owing to the difficulty of analyzing and keeping a sufficiently high number of mutagenized lines of animals.

Results: We have developed the methods necessary to perform large-scale saturation screens for mutations affecting embryogenesis in the zebrafish, Danio (Brachydanio) rerio. Firstly, a new aquarium system was developed to raise and keep large numbers of strains of genetically different fish safely and with little maintenance care. Secondly, by placing adult male fish in water containing the chemical mutagen, ethynitrosourea, we induced point mutations in pre-meiotic germ cells with a rate of one to three mutations per locus per 1000 mutagenized haploid genomes. This rate, which is similar to the mutagenesis rates produced by ethylmethanesulfonate in Drosophila, was determined for alleles at four different pigmentation genes. Finally, in a pilot screen in which mutagenized fish were screened for two generations and scored for embryonic mutants, we isolated 100 recessive mutations with phenotypes visible in the homozygous embryos.

Conclusion: The high rate of induction and recovery of point mutations, in addition to an efficient aquarium system to house large numbers of mutagenized lines, means that it is now possible to perform saturation mutagenesis screens in a vertebrate, similar to those done in invertebrates.

Current Biology 1994, 4:189–202
ENU: *N*-ethyl-*N*-nitrosourea

- Alkylation agent: transfers its methyl group to nucleotides.
- ENU was found to be more mutagenic in zebrafish than EMS.
- Pre-meiotic germ cells (spermatogonia) are mutagenized, not sperm.
- If mature sperm were mutagenized, mutations are not fixed, and progeny are mosaic.
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.
Traditional positional cloning in zebrafish

1. Mapping: Identify DNA segments ("markers") linked to mutant locus.
   - simple sequence length polymorphisms (SSLPs)
   - also called CA-repeats, SSRs (simple sequence repeats), microsatellites.
   - length of the CA tract differs in different strains

```
CACACACACACA
\[\text{200 bp}\]
```

```
CACACACACACACACA
\[\text{206 bp}\]
```
8 different SSLP markers scored on pools of WT and mutant embryos:
Traditional positional cloning in zebrafish

2. Correlate markers with genomic maps
   – ID “the critical region” containing mutant locus
Traditional positional cloning in zebrafish

2. Correlate markers with genomic maps
   – ID “the critical region” containing mutant locus
Traditional positional cloning in zebrafish

2. Correlate markers with genomic maps
   – ID “the critical region” containing mutant locus
Traditional positional cloning in zebrafish

And many markers are scored in individuals to continue to narrow the region

- **Wild type**
  - Marker 1: 2 recombinants
- **Mutant**
  - Marker 2: 0 recombinants
  - Marker 3: 2 recombinants
Traditional positional cloning in zebrafish

2. Correlate markers with genomic maps
   – ID “the critical region” containing mutant locus
With the advent of deep sequencing, not done this way anymore...

Efficient Mapping and Cloning of Mutations in Zebrafish by Low-Coverage Whole-Genome Sequencing

Margot E. Bowen,*†,1 Katrin Henke,*†,1 Kellee R. Siegfried,† Matthew L. Warman,*† and Matthew P. Harris*†,2

*Orthopedic Research Laboratories, Children’s Hospital, Boston, Massachusetts 02115, and †Department of Genetics, Harvard Medical School, Boston, Massachusetts 02115

ABSTRACT The generation and analysis of mutants in zebrafish has been instrumental in defining the genetic regulation of vertebrate development, physiology, and disease. However, identifying the genetic changes that underlie mutant phenotypes remains a significant bottleneck in the analysis of mutants. Whole-genome sequencing has recently emerged as a fast and efficient approach for identifying mutations in nonvertebrate model organisms. However, this approach has not been applied to zebrafish due to the complicating factors of having a large genome and lack of fully inbred lines. Here we provide a method for efficiently mapping and detecting mutations in zebrafish using these new parallel sequencing technologies. This method utilizes an extensive reference SNP database to define regions of homozygosity-by-descent by low coverage, whole-genome sequencing of pooled DNA from only a limited number of mutant F2 fish. With this approach we mapped each of the five different zebrafish mutants we sequenced and identified likely causative nonsense mutations in two and candidate mutations in the remainder. Furthermore, we provide evidence that one of the identified mutations, a nonsense mutation in bmp1a, underlies the welded mutant phenotype.
Whole Genome Sequencing to identify causative lesions

Amy Herbert, Kelly Monk Lab
Whole Genome Sequencing to identify causative lesions

Amy Herbert, Kelly Monk Lab
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
Recent advances in genome editing allow rapid mutant generation

- Zinc fingers
- TALENs
- CRISPR/Cas
Zinc fingers

- Fusion
  - FokI restriction enzyme
  - 3+ zinc-finger motifs to recognize DNA target sequence.

- FokI dimer -> dsDNA break

- DNA break repair mechanisms can be mutagenic

- Design difficult
  - Off-target binding
  - Poorly accessible chromatin domains
TALENs

- **Transcription Activator-Like Effector Nucleases.**

- Similar to ZFNs,
  - simpler, more reliable with fewer off-target effects.

- From plant pathogens, secreted by *Xanthomonas* bacteria during infection.
  - TALE = transcriptional activators that bind and regulate genes that aid bacterial infection
TALENs

- One 34 amino acid repeat binds 1 nucleotide.
- Repeat Variable Diresidue (RVD, positions 12 & 13) determines specificity
- Where the repeat actually binds the nucleotide.
TALENs

- Dimerization of FokI triggers DNA cleavage.
- Trigger DNA repair mechanisms which are imperfect
  - insertions/deletions.
- Co-inject DNA sequence of interest (e.g. GFP, human mutation) to introduce the sequence into the genome.
TALENs in zebrafish

Targeted gene disruption in somatic zebrafish cells using engineered TALENs

Jeffry D Sander, Lindsay Cade, Cyd Khayter, Deepak Reyon, Randall T Peterson, J Keith Joung & Jing-Ruey J. Yeh

Affiliations | Corresponding authors

Nature Biotechnology 29, 697–698 (2011) | doi:10.1038/nbt.1934
Published online 05 August 2011

Heritable gene targeting in zebrafish using customized TALENs

Peng Huang, An Xiao, Mingguo Zhou, Zuoyan Zhu, Shuo Lin & Bo Zhang

Affiliations | Corresponding authors

Published online 05 August 2011
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/Cas9 System

- Will skip more mechanistic details here…
CRISPR/Cas in zebrafish

BRIEF COMMUNICATIONS

Efficient genome editing in zebrafish using a CRISPR-Cas system

Woong Y Hwang1,7, Yanfang Fu2,3,7, Deepak Reyon2,3, Morgan L Maeder4,4, Shengdar Q Tsai2,3, Jeffry D Sander2,3, Randall T Peterson1,5,6, J-R Joanna Yeh1,5 & J Keith Joung2,4

In bacteria, foreign nucleic acids are silenced by clustered, regularly interspaced, short palindromic repeats (CRISPR)–CRISPR-associated (Cas) systems. Bacterial type II CRISPR systems have been adapted to create guide RNAs that direct site-specific DNA cleavage by the Cas9 endonuclease in cultured cells. Here we show that the CRISPR-Cas system functions in vivo to induce targeted genetic modifications in zebrafish embryos with efficiencies similar to those obtained using zinc finger nucleases and transcription activator–like effector nucleases.

complementary to a target site (Fig. 1c). The sequence of our sgRNA, like that of another recently described6, differs from a sgRNA used in vitro6 in that our sgRNA contains additional tracrRNA-derived sequences at its 3’ end (Fig. 1b,c and Supplementary Table 1). For initial experiments, we designed a sgRNA with a targeting region complementary to a sequence in the fh gene (site no. 1) (Supplementary Table 2).

To determine the optimal quantity of each RNA species to use for genome editing, we microinjected varying amounts of fh-targeted sgRNA and Cas9-encoding mRNA into one-cell-stage zebrafish embryos; we then assessed the frequency of altered alleles in single embryos using a T7 endonuclease 1 (T7EI) assay (Supplementary Methods). We observed targeted insertion/deletion mutations (indels) at all concentrations of RNAs examined and in nearly all embryos tested (Supplementary Table 3). However, the highest mean frequency of mutations was obtained with a solution containing 12.5 ng/μl sgRNA and 300 ng/μl Cas9-encoding mRNA (Supplementary Table 3), so we used these concentrations for all subsequent experiments. Sequencing of mutated fh alleles revealed indels that begin within or encompass the 5’ end of the DNA sequence complementary to the sgRNA (Supplementary Fig. 1). This pattern of mutations is consistent with the expected induction of a Cas9-induced double-stranded break at this position6 within the genomic fh target site followed by error-prone nonhomologous end joining–mediated repair.
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
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

Shared exposure

Oncogene Activation and/or Tumor Suppressor Loss
The emergence of cancer

Normal Tissue

Cancer-prone cells or
“cancerized field”

Cancer Initiation

Tumor Expansion

Shared exposure

Oncogene
Activation
and/or
Tumor
Suppressor Loss

?
The emergence of melanoma
The emergence of melanoma

Cancer-prone cells or “cancerized field”

Nevus/Mole

BRAF\textsuperscript{V600E}

Cancer Initiation

Tumor Expansion

Melanoma

BRAF\textsuperscript{V600E}

p53 loss

TERT increase

others
Melanoma Skin Cancer

- Significant cause of cancer death
  - >10,000 deaths in US in 2016 (SEER data)

- Increasing incidence
  - rate increasing 1.4% per year

- Late recurrences and poor prognosis in metastatic disease
The emergence of melanoma

Field Cancerization
Nevus/Mole

Cancer Initiation

Tumor Expansion
Melanoma

- Interrogate and modulate this process
- Animal model
Zebrafish melanoma model

- Tg(mitf:BRAG\textsuperscript{V600E}), p53\textsuperscript{LOF/LOF}
  
  Patton et al, Current Biology, 2005

- mitf:BRAG\textsuperscript{V600E}
  - injection of linearized, naked DNA
  - Random integration, concatemers, low efficiency (~1% germ line)

- p53
  - Isolated from TILLING approach
Zebrafish melanoma model

- Tg($mitf$:BRAF$^{V600E}$), p53 mutant
- responsive to oral $BRAF$ inhibitor therapy

Vemurafenib
PO
14 days

~80% tumor shrinkage

M. Dang
Zebrafish melanoma model

- Tg(*mitf:BRAF^V600E*), *p53* mutant
- responsive to *BRAF* inhibitor therapy
- invasive, histologically similar to human melanoma
Zebrafish melanoma model

- Tg\(mitf:BRAF^{V600E}\), \(p53\) mutant
- responsive to \(BRAF\) inhibitor therapy
- invasive, histologically similar to human melanoma
- molecularly similar to human melanoma

GSEA
zebrafish:human melanoma

Zebrafish melanoma model

- Tg(*mitf:BRAF^600E*), p53 mutant
- responsive to *BRAF* inhibitor therapy
- invasive, histologically similar to human melanoma
- molecularly similar to human melanoma
- metastatic potential

![Image of Zebrafish with melanoma lesions and injection site]

E. van Rooijen
The emergence of melanoma

Field Cancerization

Nevus/Mole

Cancer Initiation

Tumor Expansion

Melanoma

Interrogate and modulate this process

Animal model
The emergence of melanoma

Field Cancerization  
Cancer Initiation  
Tumor Expansion

\[ \text{BRAF}^{V600E} \]  
\[ p53 \text{ loss} \]

1-3 tumors per fish
The emergence of melanoma

Field Cancerization → Cancer Initiation → Tumor Expansion

- BRAF^{V600E}
- p53 loss

1-3 tumors per fish
Monitoring melanoma initiation \textit{in vivo}

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

Neural Crest

\textbf{CRESTIN}

\textbf{ON}
Monitoring melanoma initiation *in vivo*

The *crestin* gene:  
i) zebrafish embryonic neural crest marker

Adapted White et al., 2011
Monitoring melanoma initiation *in vivo*

The *crestin* gene:  
1) zebrafish embryonic neural crest marker  
2) melanoma “tumor sensor”?

Adapted White et al., 2011
Hypothesis: Neural crest identity arises, or reemerges, early during melanoma formation
Hypothesis: Neural crest identity arises, or reemerges, early during melanoma formation

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

\textbf{BRAF}^{V600E}, \textbf{p53} loss

1-3 tumors per fish
The *crestin* gene

- Zebrafish multicopy retroelement with no known function

![Diagram of the *crestin* gene](image)

w/ C. Mosimann
The *crestin* gene

- Zebrafish multicopy retroelement with no known function

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

Kristen M. Kwan,¹* Esther Fujimoto,¹ Clemens Grabher,² Benjamin D. Mangum,¹ Melissa E. Hardy,¹ Douglas S. Campbell,¹ John M. Parant,³ H. Joseph Yost,³ John P. Kanki,² and Chi-Bin Chien¹,4*
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

w/ C. Mosimann
The *crestin* gene

- Zebrafish multicopy retroelement with no known function
The *crestin* gene

- Zebrafish multicopy retroelement with no known function

w/ C. Mosimann
*crestin:*EGFP - neural crest reporter

24 hpf Stable *Tg(crestin:EGFP)* line

crestin ISH
Emergence and migration of neural crest – live imaging with crestin:EGFP
Lineage tracing of neural crest descendants with the *crestin* promoter

*Embryo*

Dorsal melanocyte

Eye melanocytes

Jaw cartilage

*Tg(crestin:creERt2;cryst:YFP); Tg(ubi:EGFP->mCh)*

- All *Tol2 Kit*-derived transgenics
Conclusions

• *crestin* drives transgene expression in neural crest progenitor cells during embryogenesis

*Expression in melanoma tumors?*
crestin:EGFP - a highly specific melanoma tumor sensor
**crestin:**EGFP - longitudinal tracking of melanoma tumor formation

6 wks  |  9 wks  |  11.5 wks  |  17 wks

p53/BRAF/Na/MCR/crestin:EGFP
**crestin:**\textit{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 \textit{p53/BRAF} are EGFP (+)
Conclusions

• *crestin* drives transgene expression in neural crest progenitor cells during embryogenesis

• *crestin:EGFP* specifically marks melanoma tumors and early lesions, which have tumorigenic features
Conclusions

• *crestin* drives transgene expression in neural crest progenitor cells during embryogenesis

• *crestin:EGFP* specifically marks melanoma tumors and early lesions, which have tumorigenic features

*How early?*
crestin:EGFP - visualizing melanoma initiation at a single cell level in vivo
**crestin:EGFP** - visualizing melanoma initiation at a single cell level *in vivo*

- earliest visualization of *in vivo* melanoma initiation
Conclusions

• *crestin* drives transgene expression in neural crest progenitor cells during embryogenesis

• *crestin*:EGFP specifically marks melanoma tumors and early lesions, which have tumorigenic features

• *crestin*:EGFP reveals melanoma initiation at a single cell level
Barriers to melanoma initiation

Modulate neural crest progenitor identity

BRAFV600E
p53 -/-

Neural crest progenitor state = crestin ON

Prediction:
- Favor neural crest state \( \uparrow \) melanoma formation
- Inhibit neural crest state \( \downarrow \) melanoma formation

Oncogene activation
Tumor suppressor loss
Reprogramming?
Neural crest progenitor identity and melanoma – super-enhancers at endogenous *crestin*

- Zebrafish melanoma cell line and primary tumor
Neural crest progenitor identity and melanoma – super-enhancers at endogenous *crestin*

- ATAC-seq – corresponding regions of open chromatin
Neural crest progenitor identity and melanoma – super-enhancers at sox10 in zebrafish melanoma
Neural crest progenitor identity and melanoma – super-enhancers at sox10 in zebrafish melanoma
In human melanoma, super-enhancers are present at many early melanoma genes identified in our fish model.

A375 human melanoma cells

w/ Zi Fan,
Rick Young
Conclusions – Melanoma initiation

Oncogene activation
Tumor suppressor loss

BRAFV600E
p53 -/-

Neural crest progenitor state = crestin ON
Conclusions – Melanoma initiation

Oncogene activation
Tumor suppressor loss

BRAFV600E
p53 -/-

Reemergence of Neural crest state through regulation by SE’s

Neural crest progenitor state = crestin ON
Conclusions – Melanoma initiation

Combinatorial control

↑SOX10, ↑DLX2, ↑others

BRAFV600E
p53 -/-

Neural crest progenitor state = crestin ON

Oncogene activation
Tumor suppressor loss

Reemergence of Neural crest state through regulation by SE’s

Conclusions – Melanoma initiation
Current and Future Approaches

1. *crestin:EGFP* specifically marks melanoma initiation with single cell resolution
Current and Future Approaches

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*Unique opportunity to better profile the earliest lesions*
Current and Future Approaches

1. *crestin:EGFP* specifically marks melanoma initiation with single cell resolution

   Unique opportunity to better profile the earliest lesions

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*Melanoma*

mitf:BRAF$^\text{V600E}$

p53 -/

*crestin:EGFP*
Current and Future Approaches

2. Regulation of neural crest progenitor identity in melanoma is linked to super-enhancers (SE’s)
Current and Future Approaches

2. Regulation of neural crest progenitor identity in melanoma is linked to super-enhancers (SE’s)
   - Forward genetic screen for new neural crest regulators (in collab with Lila Solnica-Krezel lab)

   - F3 embryos – ideal result
Current and Future Approaches

2. Regulation of neural crest progenitor identity in melanoma is linked to super-enhancers (SE’s)
   - Affect melanoma initiation through modification of the chromatin landscape
     - Delete enhancer elements using CRISPR/Cas9

Melanoma
mitf:BRAF^{V600E}
p53 -/-
crestin:EGFP

Chromatin State
Current and Future Approaches

2. Regulation of neural crest progenitor identity in melanoma is linked to super-enhancers (SE’s)
   - Affect melanoma initiation through modification of the chromatin landscape
     - Use functionalized dCas9 to alter chromatin landscape

Melanoma
mitf:BRAF^{V600E}
p53 -/-
crestin:EGFP

dCas9
Chromatin State
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