Zebrfish genetics: Fish and ChIPs and beyond

February 13, 2020
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The emergence of cancer

Normal Tissue → Cancer-prone cells or “cancerized field” → Cancer Initiation → Tumor Expansion
The emergence of cancer

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

“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
The emergence of cancer

Normal Tissue

Cancer-prone cells or “cancerized field”

Cancer Initiation

Tumor Expansion

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
Additional mutations? 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

Medaka
Why zebrafish?

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

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Why zebrafish?

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Why zebrafish?

• Goal of developing a genetically tractable vertebrate model system
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

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:

gamma-ray

(VE 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

Normal diploid = d
Normal haploid = A
Abnormal haploid = B, C

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

= nodal

= brachyury

= aT-box transcription factor
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.
Efficient Mapping and Cloning of Mutations in Zebrafish by Low-Coverage Whole-Genome Sequencing

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

*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 bmp7a, 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.

Genetic compensation induced by deleterious mutations but not gene knockdowns

Andrea Rossi*, Zacharias Kontarakis*, Claudia Gernt, Hendrik Nothe, Sosyta Hölper, Marcus Krüger† & Didier Y. R. Stainier†
Updated guidelines one should follow
https://journals.plos.org/plosgenetics/article?id=10.1371/journal.pgen.1007000
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

Woong Y Hwang1,7, Yanfang Fu2,3,7, Deepak Reyon2,3, Morgan L Maeder2,4, Shengdar Q Tsai2,3, Jeffry D Sander2,3, Randall T Peterson1,3,6, 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.

The sequence of our sgRNA, like that of another recently described, differs from a sgRNA used in vivo in that our sgRNA contains additional tracrRNA-derived sequences at its 3' end (Fig. 1bc and Supplementary Table 1). For initial experiments, we designed a sgRNA with a targeting region complementary to a sequence in the fht-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 fht-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 fht 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 position within the genomic fht target site followed by error-prone non-homologous 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
TEAZ: Transgenic Electroporation of Adult Zebrafish

Adapted from Callahan et al., 2018
Versatility of TEAZ: skin and melanoma model

Callahan et al., 2018
Zebrafish in practice - chemical genetics and precision medicine

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

Dong Li, Michael E. March, Alvaro Gutierrez-Uzquiza, Charly Ko, Christoph Seiler, Erin Pinto, Leticia S. Matsuoka, Mark R. Battig, Elizabeth J. Bhoj, Tara L. Wenger, Lifeng Tian, Nora Robinson, Tiancheng Wang, Yichuan Liu, Brant M. Weinstein, Matthew Swift, Hyun Min Jung, Courtney N. Kaminski, Rosetta Chiavacci, Jonathan A. Perkins, Michael A. Levine, Patrick M. A. Sleiman, Patricia J. Hicks, Janet T. Strausbaugh, Jean B. Belasco, Yoav Dor and Hakon Hakonarson

Altered transcription
Zebrafish in practice - chemical genetics and precision medicine

- Generalized lymphatic anomaly (GLA) –
  - multifocal lymphatic anomaly
  - multiple areas of micro/macrocytic 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
- p53 loss
- TERT increase
- Others
The emergence of melanoma - an epigenetic phenomenon?

1 - 3 tumors per fish

Melanocyte

Nevus/Mole

Melanoma

BRAF<sub>V600E</sub>

p53 loss

>10,000’s melanocytes

1-3 tumors per fish
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

Balch et al, JCO, 2009
Fig. 1. Clinical images of melanomas. Subtypes of melanoma include superficial spreading melanoma (A), amelanotic melanoma (B), nodular melanoma (C), acral lentiginous melanoma (D), and uveal melanoma (E). Images courtesy of H. Tsao, C.H. Won, and I. Kim.
Why wear sunscreen?
Why wear sunscreen?

The prevalence of somatic mutations across human cancer types.

Melanoma Skin Cancer

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

Cancer Genome Atlas Network, Cell, 2015
Melanoma Skin Cancer

• Recurrent driver mutations (cutaneous)
  – *BRAF, RAS, NF1, 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

Adapted, Lo and Fisher, Science, 2014
Zebrafish melanoma model

- Tg(\textit{mitf:human BRAF}^{V600E}), \textit{p53} \textit{lof}
  
  Patton et al, Current Biology, 2005

- NRAS, HRAS, and NF-1-driven \textbf{cutaneous} models
- GNAQ/11-driven \textbf{uveal} melanoma model
- \textit{SPRED1} \textbf{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 \textit{in vivo}? 

The \textit{crestin} gene: 

1) zebrafish embryonic neural crest marker

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

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

Adapted White et al., 2011
How to monitor melanoma initiation *in vivo*?

The *crestin* gene:  

i) zebrafish embryonic neural crest marker

![Neural Crest illustration](image1)

![Adult CRESTIN OFF](image2)

![Melanoma CRESTIN ON](image3)

Adapted White et al., 2011
The emergence of melanoma

crestin:EGFP as the tumor sensor?

BRAF^{V600E}
p53 loss
The *crestin* gene

- Zebrafish multicopy retroelement with no known function
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* 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
The *crestin* gene
The *crestin* gene
creatin - neural crest marker

Stable Tg(creatin:EGFP) line

creatin ISH

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

Oncogene activation
Tumor suppressor loss

BRAFV600E
p53 -/-

Dedifferentiation?
Reprogramming?

Neural crest progenitor state = crestin ON

Conclusion:
Reemergence of neural crest identity occurs during melanoma initiation
Barriers to melanoma initiation

Modulate neural crest progenitor identity

- Oncogene activation
  - BRAFV600E
  - p53
- Tumor suppressor loss
  - Melanocyte-specific inactivation of sox10

Favor neural crest → melanoma formation
- Overexpress sox10 → Faster onset

Inhibit neural crest → melanoma formation
- Melanocyte-specific inactivation of sox10 → slower onset
Regulation of neural crest progenitor identity

Epigenetic landscape in melanoma – “regions of interest”

- Super-enhancers (SE’s) - H3K27Ac marks
  Hnisz et al, Cell, 2013

- Open chromatin domains - ATAC-Seq
  Buenrostro et al, Nat Meth, 2013

These regions of interest are near SOX10 and other neural crest genes
in human melanoma cell lines
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?
A) Upstream events driving neural crest identity reemergence and melanoma initiation

Normal Melanocyte → Nevus/Mole → Melanoma

BRAF^{V600E} p53 -/-

mitf:mCherry Melanocytes = RED

Melanoma = crestin ON/EGFP

Eva Kramer
A) Upstream events driving neural crest identity reemergence and melanoma initiation

Normal Melanocyte → Nevus/Mole → Melanoma

BRAF\textsuperscript{V600E} p53 -/-

RNA-Seq

ATAC-Seq

mitf:mCherry
Melanocytes = RED

Melanoma = crestin ON/EGFP

Eva Kramer
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
rxrGa
zeb1b/zeb1a
phf12b
wnt7b
tcf7l1
mef2cb

Martik and Bronner, Trends in Genetics 2017
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

Cunningham et al, bioRxiv, 2/6/2020
9 out of 11 open chromatin regions can drive EGFP reporter expression in neural crest cells.

Random transgene integration, genetic mosaics, founders (F0’s)
Evaluate multiple independently derived, stable reporter lines (≥F1) for:

- EGFP expression during development
- EGFP expression in melanoma

**Number Independent Stable lines:**

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<tr>
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<td>Peak 13</td>
<td>5</td>
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<td>sox10min</td>
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Evaluate multiple independently derived, stable reporter lines (≥F1) for:

- EGFP expression during development
- EGFP expression in melanoma

**Number Independent Stable lines:**

Peak 1: 1
Peak 2: 2
Peak 3: 1
Peak 2 and 3: 2
Peak 4: 3
Peak 5: 5
Peak 7: 1
Peak 8: 6 (variable)
Peak 11: 0
Peak 12: 0
Peak 13: 5
sox10min: 6
A case study: *Peak5* enhancer activity

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

- Upregulation of EGFP reporter in early (pre-clinical) melanomas
What transcription factors regulate Peak5 in neural crest and melanoma?

**Peak5**

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</tr>
<tr>
<td>Oxygymnocypris</td>
<td>CTCATTCCTGCCCTCTCCG----------T</td>
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</table>

192 of 669 bp
Using sequence conservation to guide analysis: Peak5

**B**

- WTpeak5:betaglobin:EGFP
- peak5Δ192:betaglobin:EGFP
- peak5conserved:betaglobin:EGFP

---

**Peak5 Conserved Sequence**

**crestin:mcherry**

Sophia DeGeorgia
Using sequence conservation to guide analysis: *Peak5*

*Peak5* conserved sequence:

- **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 -> *peak5Δ192* loss of GFP
Using sequence conservation to guide analysis

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<tr>
<th>Peak 2</th>
<th>Peak 3</th>
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<table>
<thead>
<tr>
<th>Peak 4</th>
<th>Peak 8</th>
<th>Sox10 min</th>
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</table>
Hypothesis: SoxE (e.g. Sox10) binding sites crucial for neural crest (and melanoma) activity of Peak5

DNase I hypersensitive sites from two human melanoma cell lines

Over-representation of SoxE dimers (SOX10 or SOX9)

Huang et al., Sci Rep, 2015
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*
How do individual open chromatin regions function in their native context at *sox10*?

**Germline Deletion!**  
*Peak5*: 540 bp deletion

**sox10min**: 200 bp deletion
How do individual open chromatin regions function in their native context at *sox10*?

Stable Line

*sox10*min: 200 bp deletion

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

Uninjected siblings

**Peak5** CRISPR Injected (F0)

Deletion of **Peak5** at native *sox10* locus alters neural crest or melanocyte function

Tumor onset rate?
Model: *Peak5* is a critical regulator of *sox10* activity in neural crest and melanocytes via autoregulation by *Sox10*.
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 peak analysis (ATAC-seq)
- Differential gene expression (RNA-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 - predict increase melanoma onset rate?
Model for sox9b action

sox9b

sox9b

 Normal melanocyte function

sox10

~17kb ~29kb
Model for sox9b action
Melanoma initiation

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

• Caloric restriction leads to longer life
  • "La vita sorba" = "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


Vadim Grigura
What about melanoma initiation?

BRAF\textsuperscript{V600E}
p53 \textendash\/\textendash

Neural crest progenitor state = crestin ON

\begin{itemize}
\item \textbf{BRAF\textsuperscript{V600E}}
\item p53 \textendash\/\textendash
\item Neural crest progenitor state = crestin ON
\end{itemize}
Feeding amount alters melanoma initiation

Unpublished 8.5th week, scored on 2/17

12_20 Cohort - Tumor patch free survival

% Patch Free Survival

4X vs 1X
p = .0009
n=25/group

Age (days)
Intermittent fasting – protective against melanoma initiation

Metabolic input to epigenetic "rewiring" during melanoma initiation?

Unpublished

Vadim Grigura
Intermittent fasting – protective against *persistence* of early patches?

Continuous feeding

Tumors that progressed

![Images: Pre-treatment vs. After 13 days feeding](image_url)
Intermittent fasting – protective against **persistence** of early patches?

Continuous feeding

Tumors that regressed

2 week fast

Tumors that progressed

Pre-treatment

After 13 days feeding

Pre-treatment

After 13 day starve
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) mediate the effect?
   A. Can we use drugs to achieve the same effect?
Time 0

After 2 week fast
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?
Fasted, Complete Regression, then 2 week refeed

Goes away, stays away...
Day 0

Day 8

Day 13

Day 23

8 day fast

13 day fast

Day 37

Partial regression -> “rebound” growth on refeed

23 day fast

14 day refeed
Integrating metabolomic analysis and genetics/epigenetics

1. What happens to these early melanoma tumors –
   Higher resolution imaging of "fasted" tumor cells

   Apoptosis? – cleaved caspace 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?
   GH/IGF axis
C. Recurrent, non-coding mutations in human melanoma

*Human genomics and the zebrafish model*

Melanoma is a highly mutated cancer
Are there mutations in non-coding (i.e. regulatory) regions that contribute to melanoma formation?

Creation (or loss) of transcription factor binding sites modulating melanoma initiation or behavior?

Modified from Bradner et al, Cell, 2017

Paula Godoy
Are there mutations in non-coding (i.e. regulatory) regions that contribute to melanoma formation?

- TERT promoter – activating somatic variant in >80% of human melanomas
  
  Huang et al, Science, 2013
  Horn et al, Science, 2013

- "Oncogenic transcriptional enhancers" in leukemia

  Mansour et al, Science, 2011
Genes expressed in human melanoma

H3K27Ac ChIP-Seq or ATAC-Seq

Human Melanoma

23,944,558 non-coding variants from 183 human melanoma samples (ICGC Data Portal)

Putative enhancer/promoter domains

Filtered out

21 genes associated with 556 recurrent non-coding variants

Functional in melanoma? Transgenic reporter analysis?

Gene	Coordinate	Wild-type	Mutant	# of donors

<table>
<thead>
<tr>
<th>Gene</th>
<th>Coordinate</th>
<th>Wild-type</th>
<th>Mutant</th>
<th># of donors</th>
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</tbody>
</table>

Updating pipeline – Incorporating allelic-specific expression data? Ignore chromatin marks?
C. Recurrent, non-coding mutations in human melanoma

*Human genomics and the zebrafish model*

- Does non-coding variant alter expression level of target gene?
  - Luciferase assay in human melanoma cell lines

TERT promoter variants – confirmed published results in human melanoma cell line (A375)
C. Recurrent, non-coding mutations in human melanoma

*Human genomics and the zebrafish model*

- Does non-coding variant alter expression level of target gene?
  - Reporter assays in zebrafish

---

**Diagram:**

- **Human TERT WT:**
  - EGFP
  - mCherry

**Legend:**

- **Variant**
  - EGFP
  - mCherry

**Question:**

*Increased* or *Decreased* activity from human variant?
C. Recurrent, non-coding mutations in human melanoma

_Human genomics and the zebrafish model_

- Does non-coding variant alter expression level of target gene?

- Does altered expression of putative target gene alter melanoma onset?
  - Overexpression or CRISPR inactivation -> melanoma growth in zebrafish
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
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Steve Cantor
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MIT Zi Fan
Rick Young

Julien Ablain
Justin Tan
Rachel Fogley
Elliott Hagedorn
Christie Ciarlo
Chris Lawrence
Isaac Adatto

UZH Christian Mosimann
MSK Richard White

NIAMS
Cancer Research Foundation
Melanoma Research Alliance
National Cancer Institute
We put the BRAF in zeBRAFish!