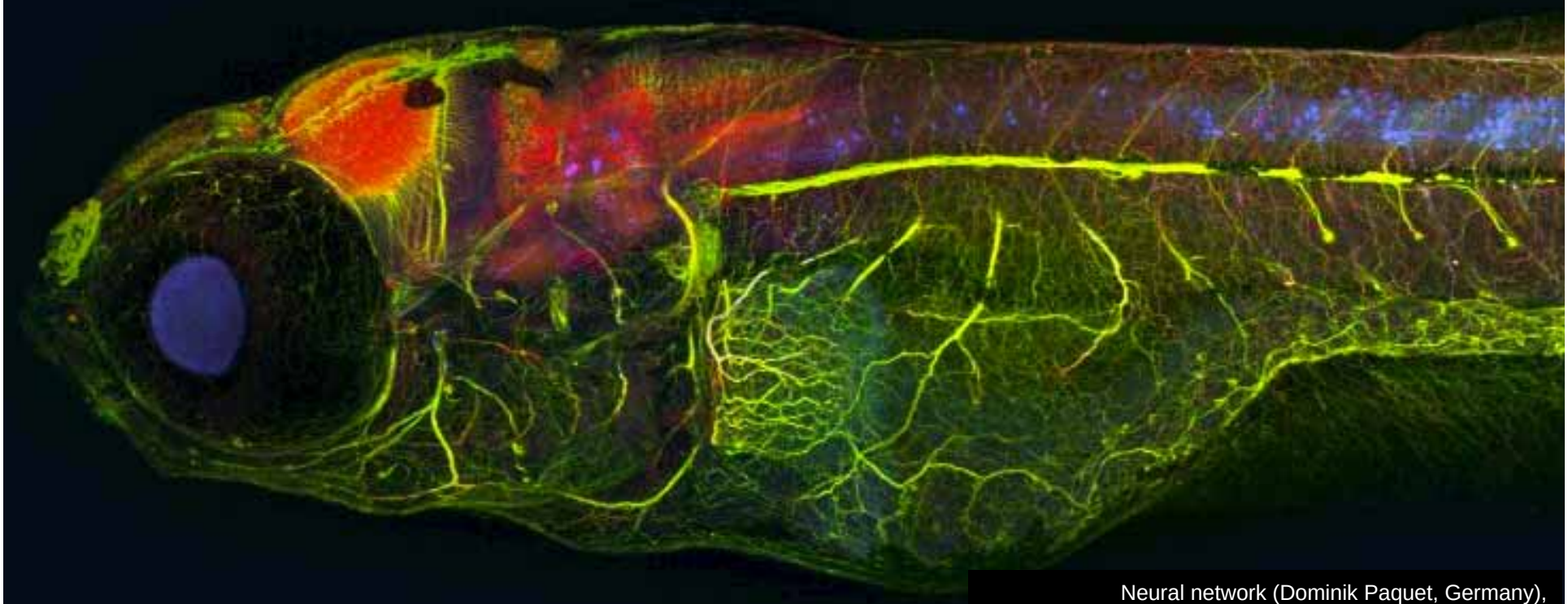
Zebrafish: a new model for human disease



Neural network (Dominik Paquet, Germany),
neurons (green), tau (red), pathologic tau (blue)

Miho Matsuda

Department of Cell Biology and Molecular Medicine

Rutgers - New Jersey Medical School

Objectives

1. To describe zebrafish genetics and technology for biomedical research

2. To demonstrate the use of zebrafish for biomedical research

Animal models in biomedical research

Biomedical research depends on the use of animal models

- To understand the pathogenesis of human disease at a cellular and molecular level

- To develop and test new medications and therapies

Mouse: Most widely used model of human disease

- Striking homology between mammalian genomes
- Similarity in basic anatomy, physiology and cell biology
- Sophisticated transgenic and gene knockout approaches

Cons: cost, time, space

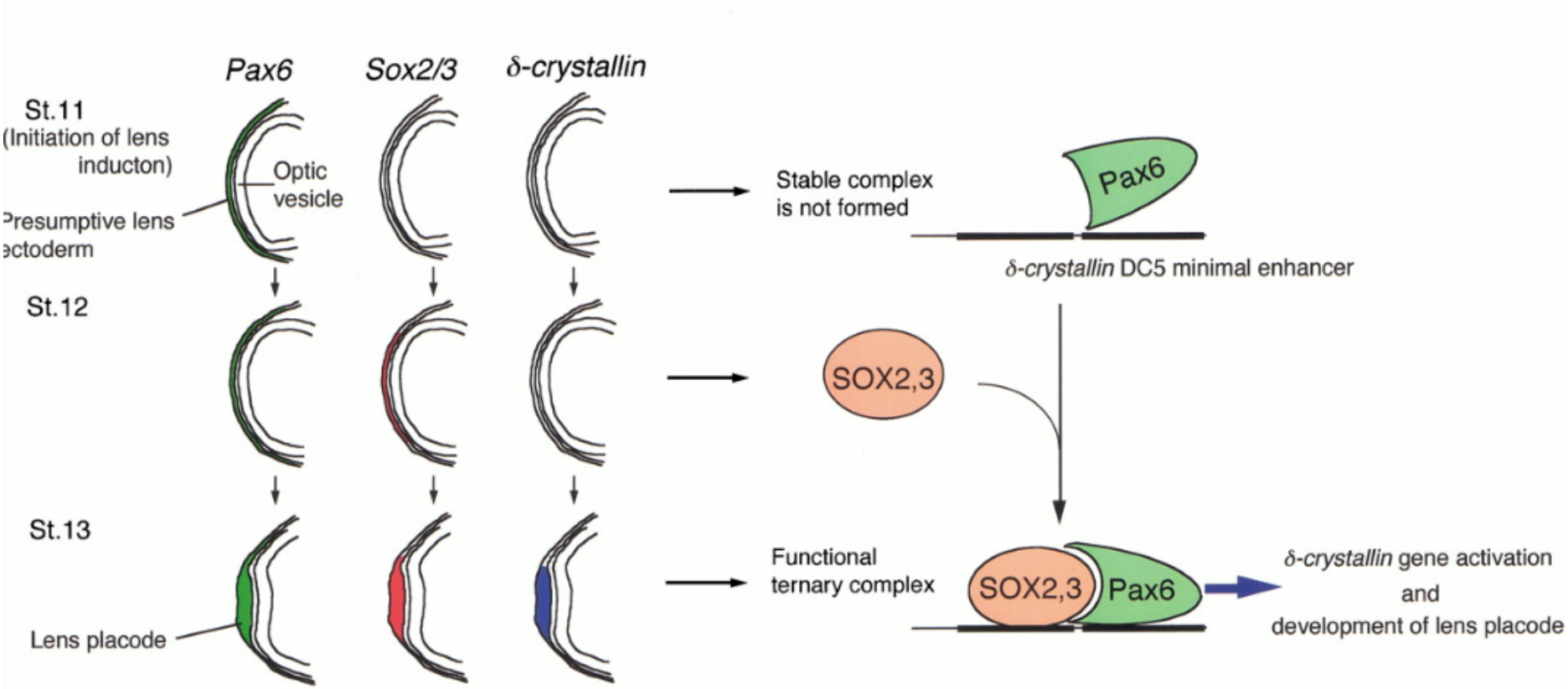
Fruit fly: invertebrate model system

- Functional conservation in basic cell biological processes between mammals and invertebrates
- Disruption of conserved cellular processes in mice can be accurately modeled in fly

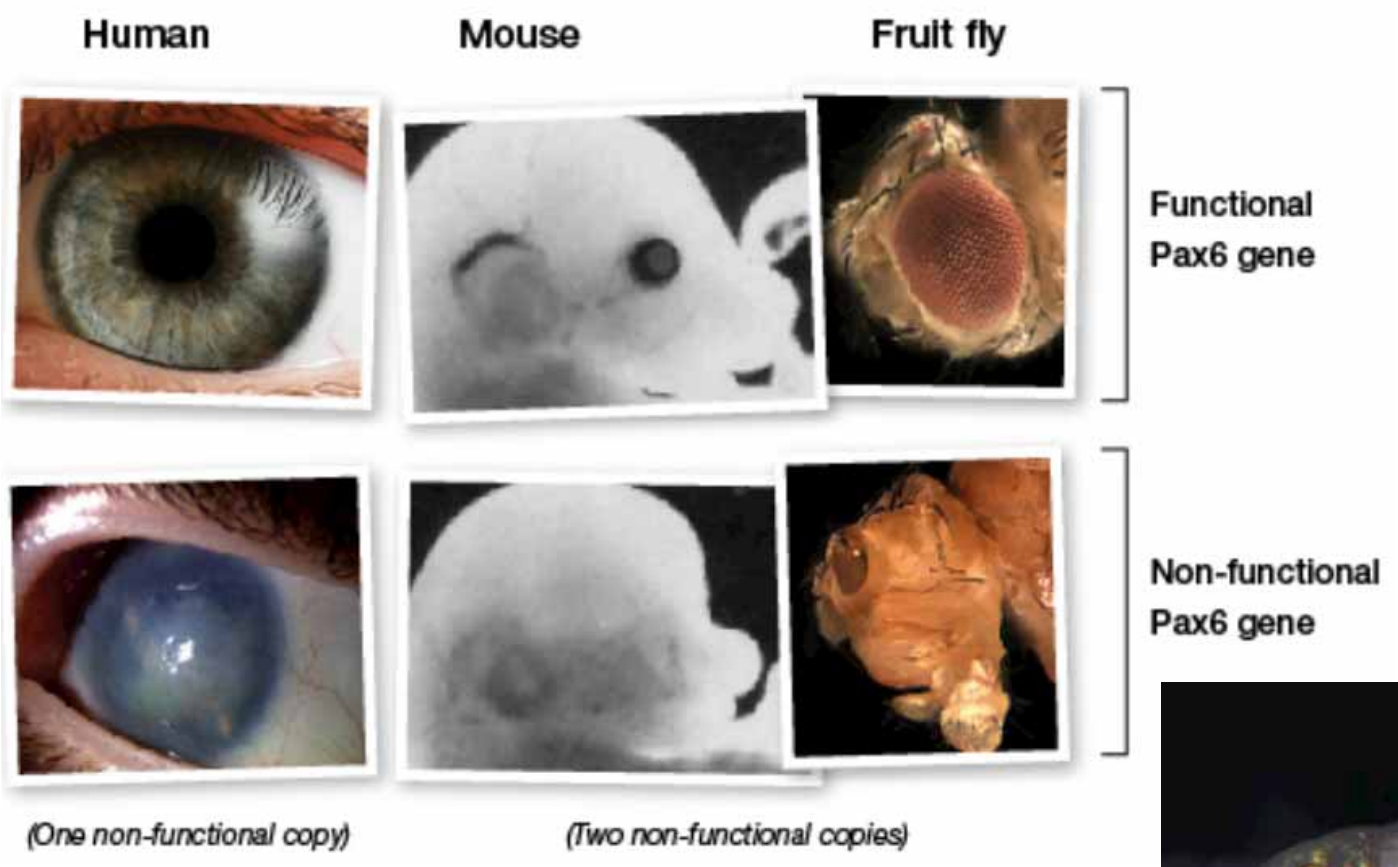
Pax6 --- A “master” transcription factor essential for eye development

A. Gene expression during lens development

B. Molecular events



Disruption of conserved cellular processes in vertebrates can be accurately modeled in fly



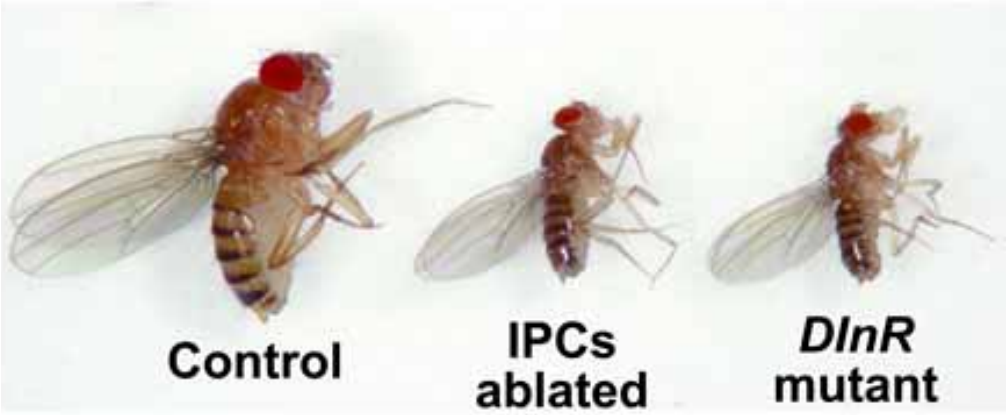
One Biology 7(11): e1000247. doi:10.1371/journal.pbio.1000247

Loss of eyes in Mexican cave fish (mutation in pax6 gene)

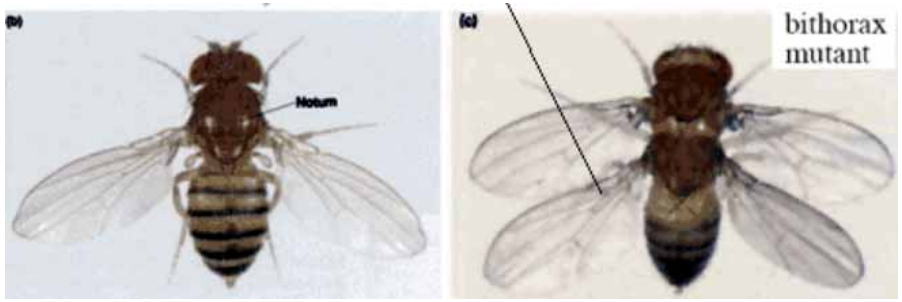
Fruit fly: invertebrate model system

- Inexpensive, Small
- Large-scale “forward-genetic” mutagenic screens provide insight into how orthologous human disease genes function in similar processes

Body size



Pattern formation (wing)



Cons: not vertebrate

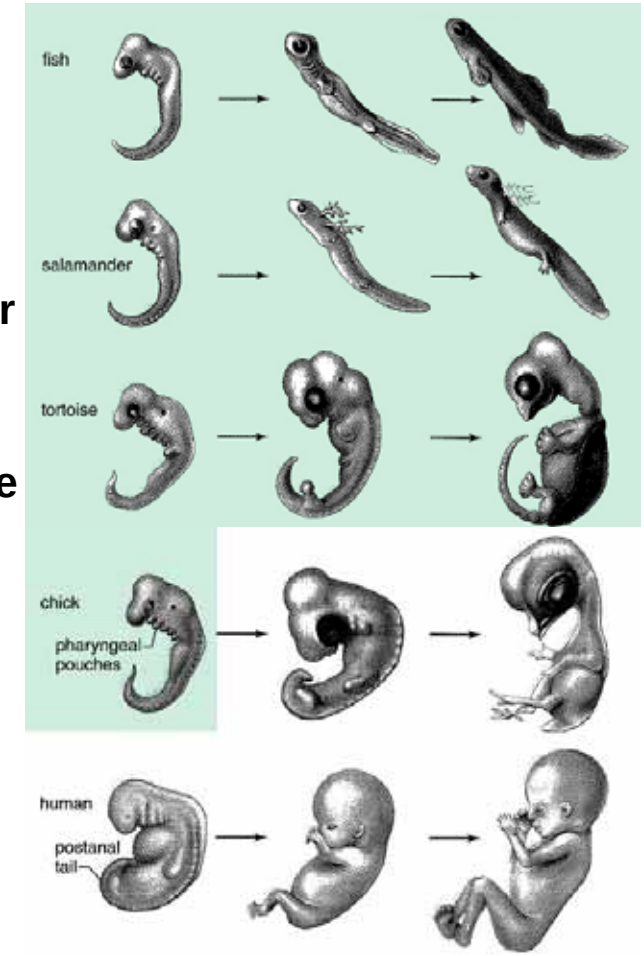
Zebrafish: an alternate vertebrate model organism to study human disease

1. Vertebrate

Significant of developmental similarities ---

- Morphological conservation
- Molecular conservation

1. Morphological conservation
2. Molecular conservation



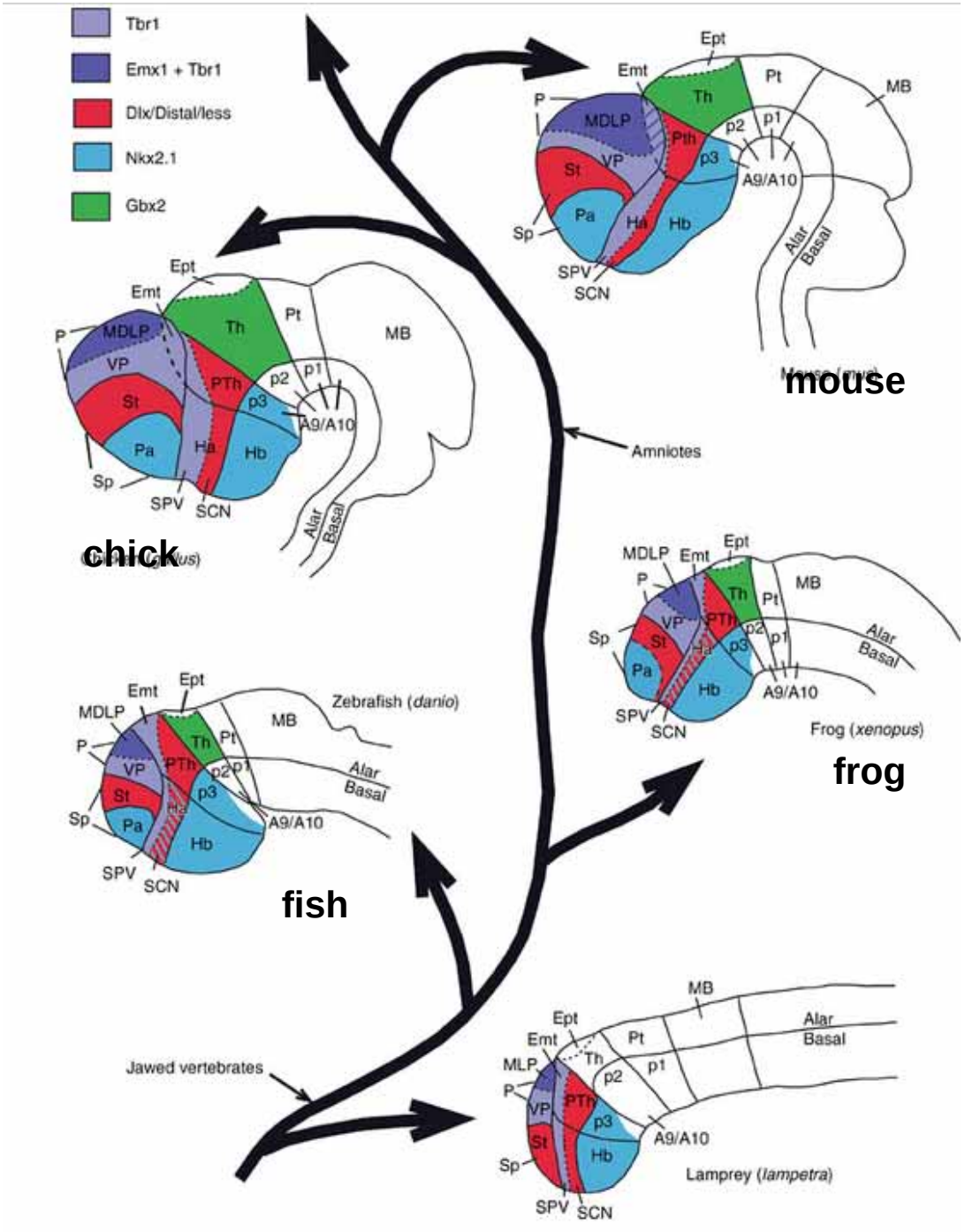
fish

sala mander

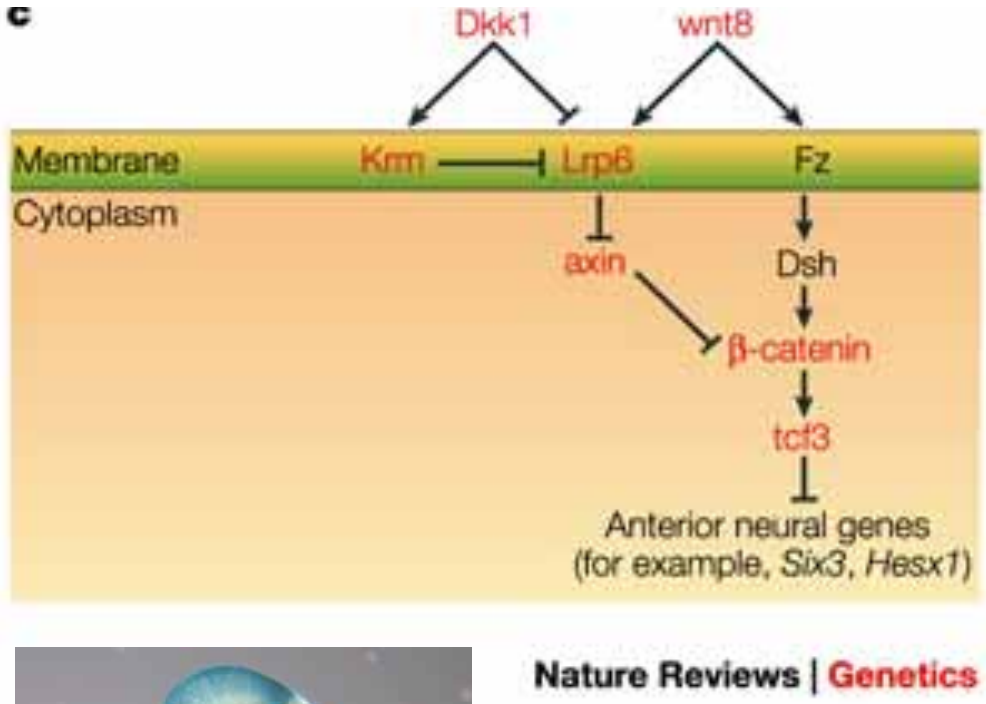
tortoise

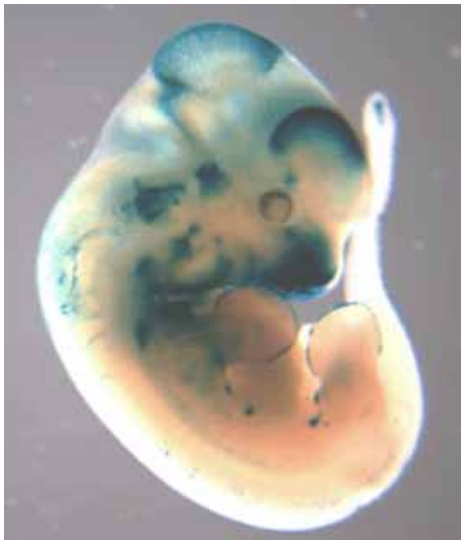
chick

human



Molecular conservation:
Forebrain formation regulated by Wnt signaling





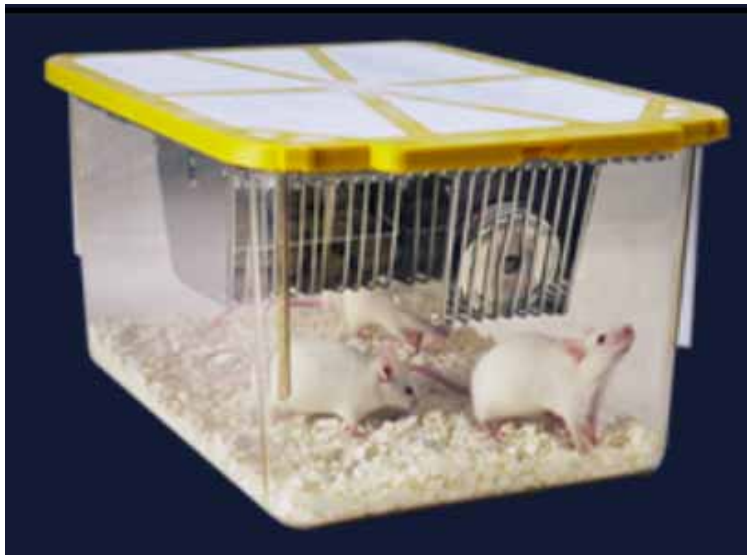
Top flash Wnt reporter mouse

zebrafish *headless* (*hdl, tcf3*) mutants

dkk1 knockout mouse

Zebrafish: an alternate vertebrate model organism to study human disease

1. Vertebrate
2. Small size, many fish in a small space
3. Relatively low maintenance cost



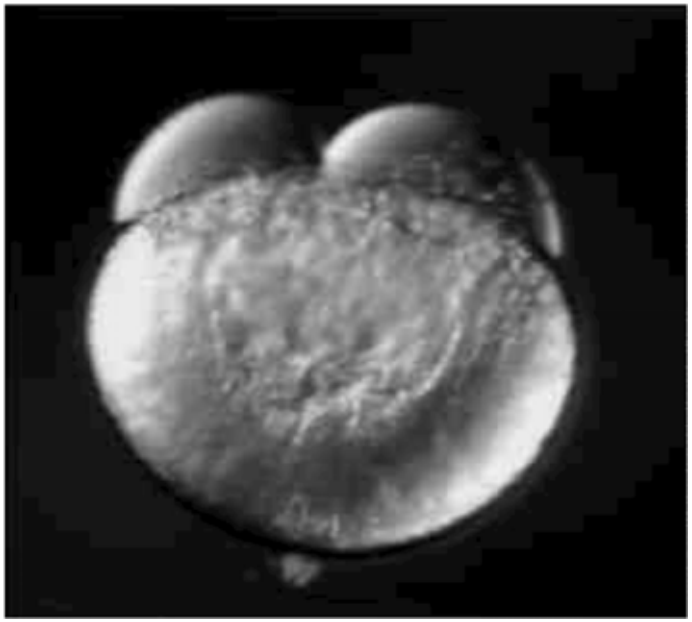
> 5 mice per 3L cage

> 24 fish per 3L tank

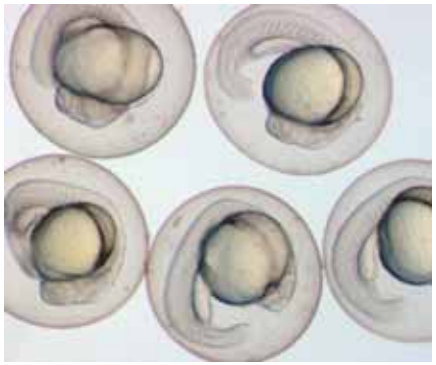
Zebrafish: an alternate vertebrate model organism to study human disease

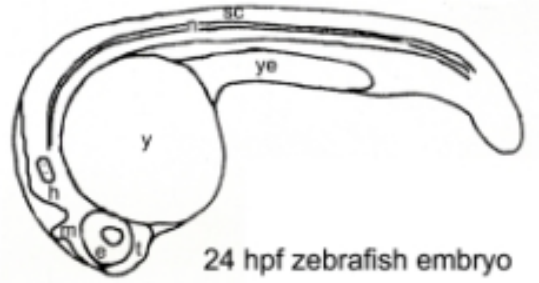
1. Vertebrate
2. Small size, many fish in a small spac
3. Low maintenance cost
4. Study of embryonic development

   external development, rapid developr



18 hours post fertilization





Zebrafish: an alternate vertebrate model organism to study human disease

1. Vertebrate
2. Small size, many fish in a small space
3. Low maintenance cost
4. Study of embryonic development
5. Large-scale “forward-genetic” screens
6. Large-scale “chemical” screens

Zebrafish are not just aquatic mice!

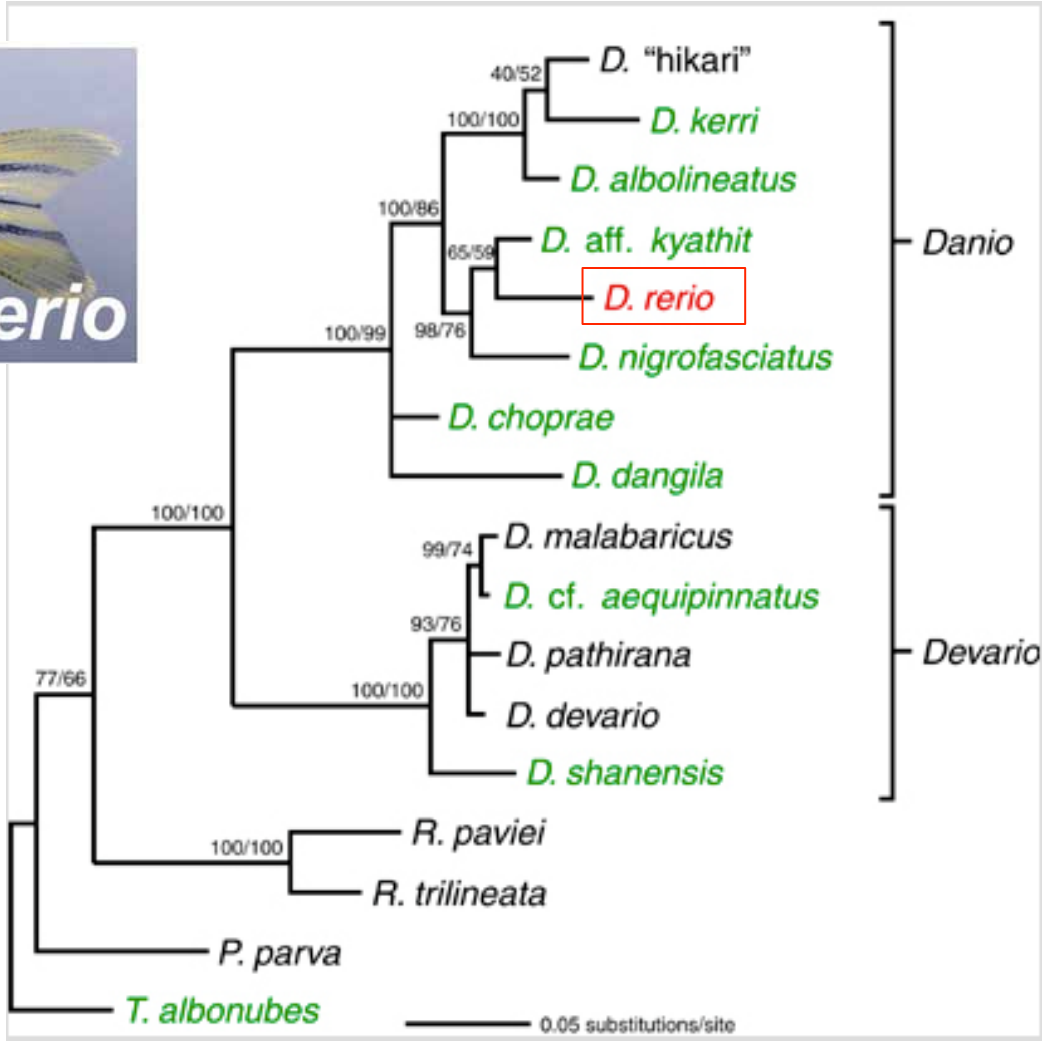
+

=

What is zebrafish, *Danio rerio*?

Blue-purple horizontal stripes run from gill to tail, "zebra"fish

Order: *Cypriniformes*
Family: *Cyprinidae*
Genus: *Danio*



Parichy, DM. *Heredity* (2006)

Zebrafish Fact Sheets

- **Small tropical fresh-water fish**
- **Average length of adult zebrafish is 1-2.5 inch**
- **Live in rivers of northern India, Pakistan and Nepal**
- **Adapt to a broad range of temperature from 42˚F to 100˚F**
- **Rapid development**

  --- all major organs are formed in 24 h after fertilization

  --- hatch and eat food within 3 days

  --- sexually mature in 3 months
- **A single female can lay up to 200 eggs**
- **25 chromosomes, 1.5 billion base pairs**
- **Zebrafish genome sequence assembly was released in 2002**

Male or Female?

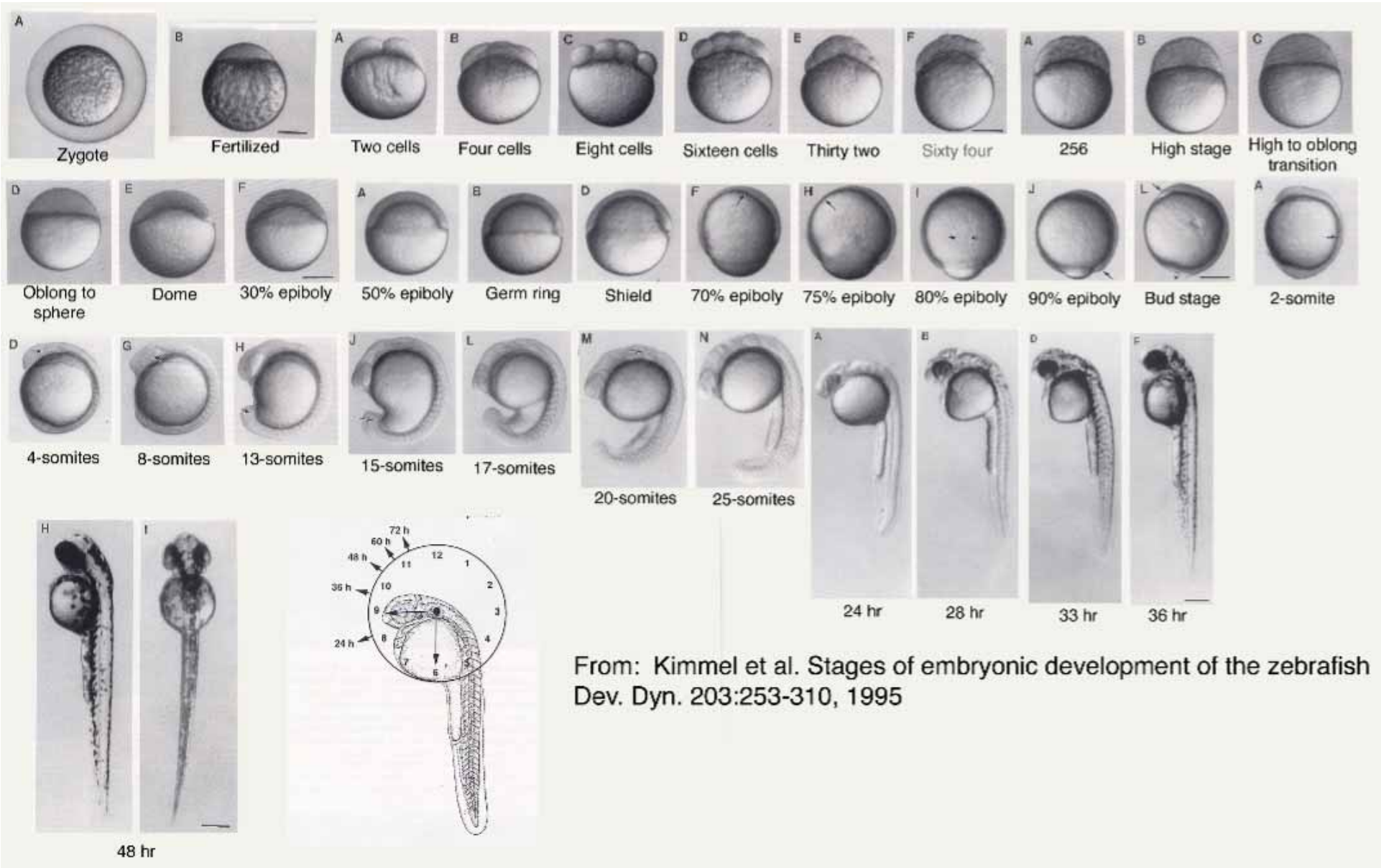
Color --- Female fish are blue and white
Male fish are pink and yellow

Body shape --- Female fish are more rounded
Male fish are slender

How to obtain zebrafish embryos?

1. Place male and female fish in a breeding tank
2. Spawning only occurs within 2-3 hours after the light comes on
3. Male fish chases female, stimulates female to spawn eggs and fertilizes eggs
4. Collect eggs in a petri dish
5. Raise embryos at 28C

Development of zebrafish embryos



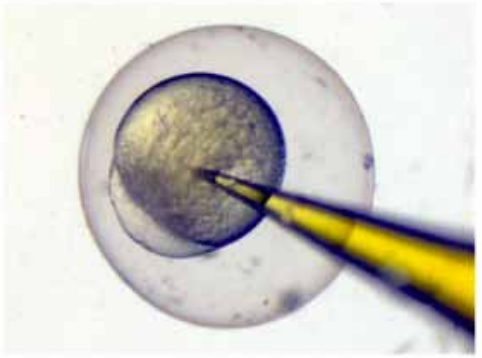
From: Kimmel et al. Stages of embryonic development of the zebrafish
Dev. Dyn. 203:253-310, 1995

How to generate zebrafish disease models

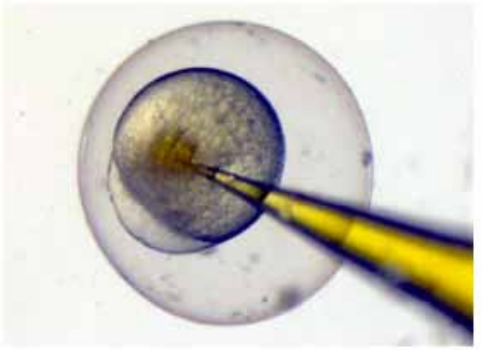
1. Loss of function ---
   a) Transient gene knockdown
   b) Generation of mutant zebrafish

2. Gain of function ---
   a) Transient mRNA overexpression
   b) Generation of transgenic zebrafish

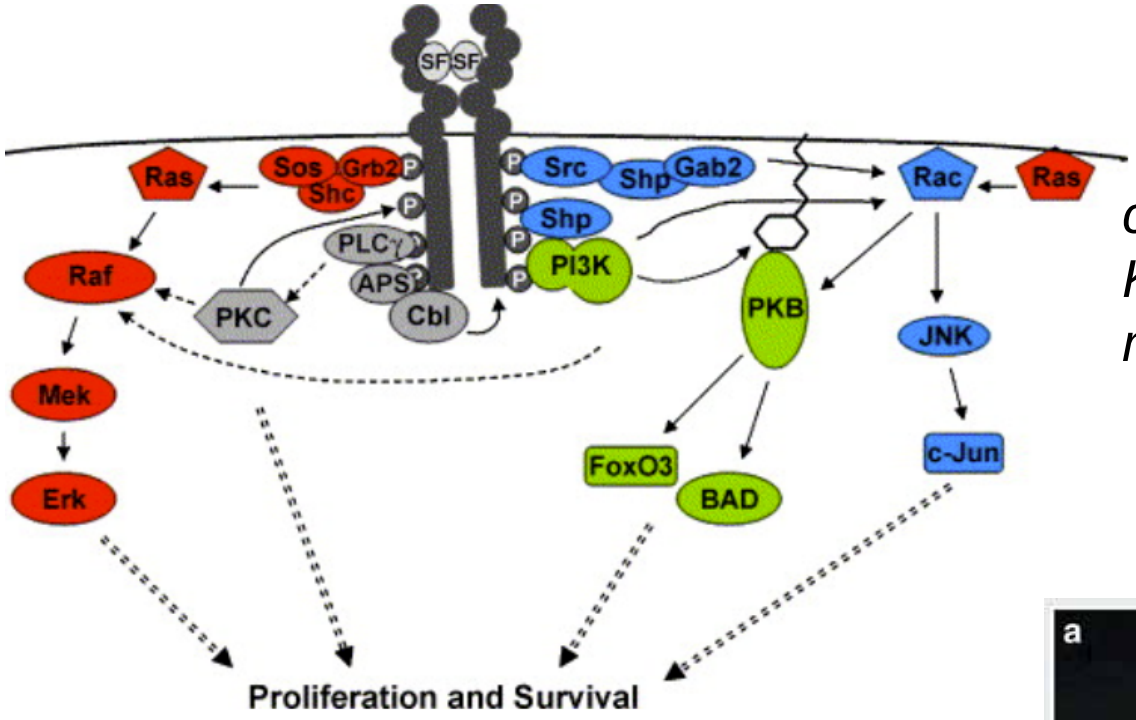
3. Drug treatment
   a) Inhibitors
   b) Activators



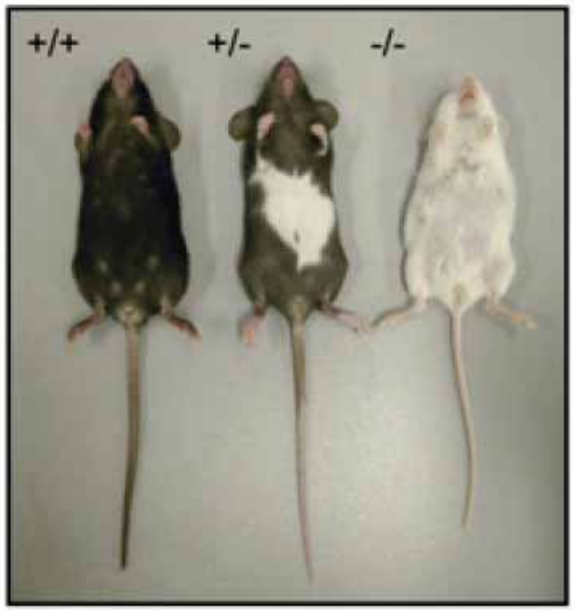
First the glass needle penetrates the chroion into yolk mass

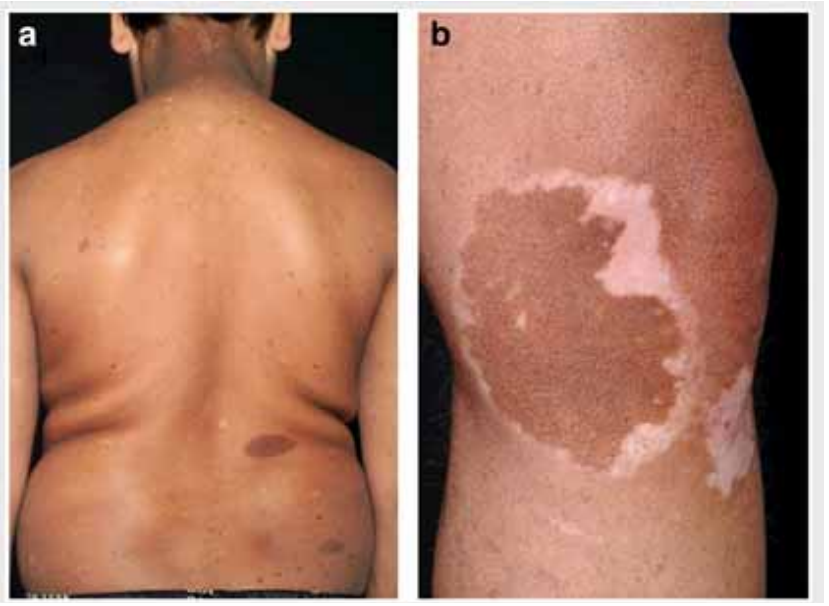


c-kit/kit ligand signaling pathway



c-kit KO mice





Journal of Investigative Dermatology (2011) **131,** 1234–1239; doi:10.1038/jid.2011.29; published online 3 March 2011

KITLG Mutations Cause Familial Progressive Hyper- and Hypopigmentation

Manipulation of gene expression in pigment cell development

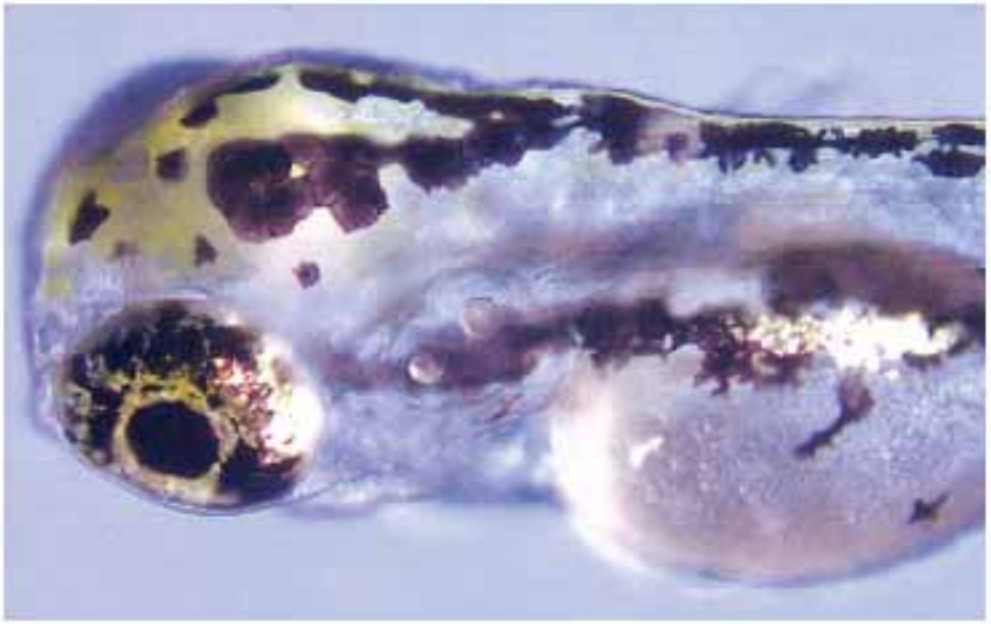


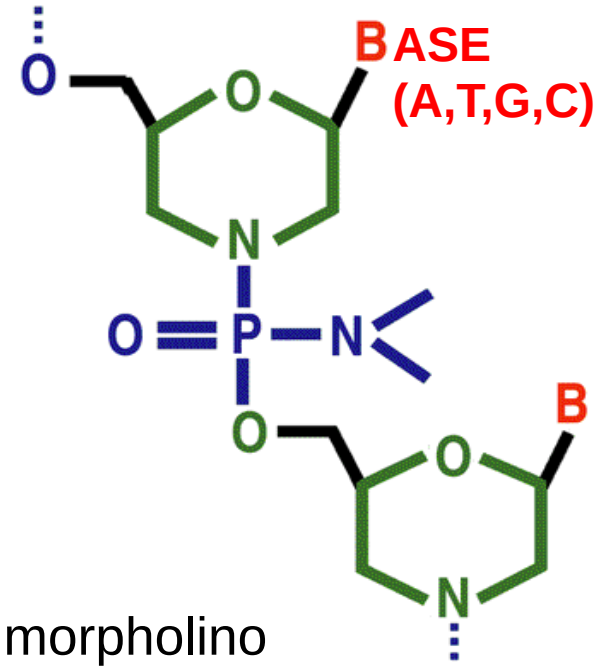
Fig. 1. Pigment cell types in the zebrafish embryo. Shown is a wild-type zebrafish larva at approximately 4 days post-fertilization, viewed from a dorsolateral position. At this stage, most of the melanophores (black pigment cells) have organized into horizontal stripes. Xanthophores give the top of the head a yellow appearance and silvery iridophores can be seen on the choroid of the eye, in bilateral patches over the yolk (one is visible in this view) and mixed in with melanophores in the stripes of the trunk and tail (not shown).

Melanophore --- black pigment cells

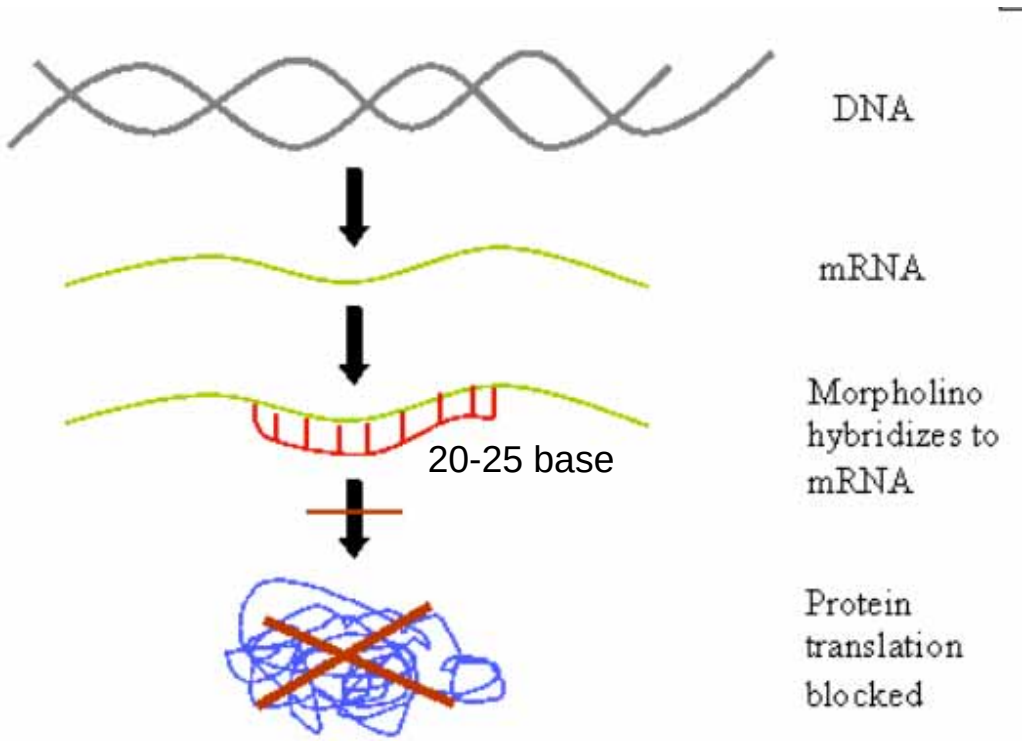
Xanthophore --- yellow pigment cells

Iridophores --- Silver pigment cells

Transient gene knockdown --- morpholinos



morpholino



- Morpholino oligos: antisense oligonucleotides
- Morpholino oligos bind to complementary sequences of mRNA by standard nucleic acid base-paring
- It blocks initiation of mRNA translation or mRNA splicing

Transient Gene overexpression --- mRNA

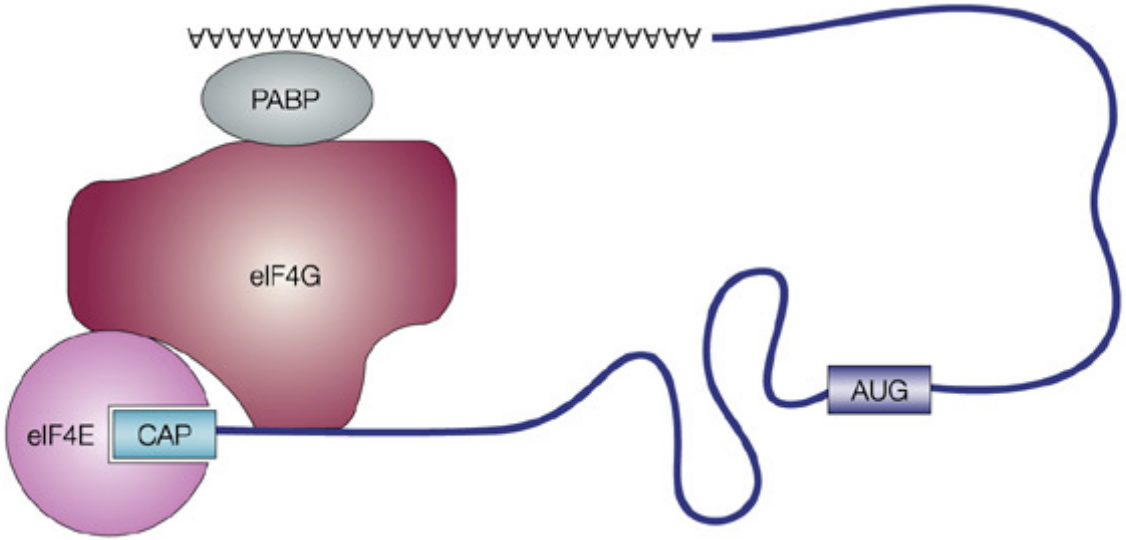
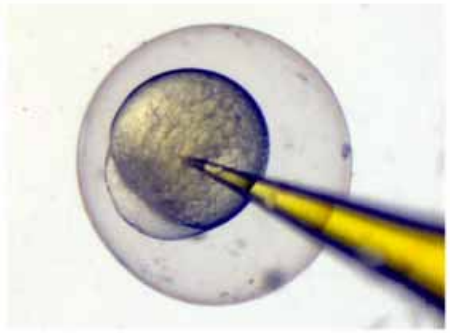


Figure 1 | mRNA circularization.
Communication between the 5' cap and the 3' poly(A) tail of mRNA results in the enhancement of translation. This occurs when the poly(A)-binding protein (PABP) interacts with the translation initiation factor eIF4G, which, in turn, interacts with the 5' cap-binding protein eIF4E, bringing the 5' and 3' ends of the mRNA together.



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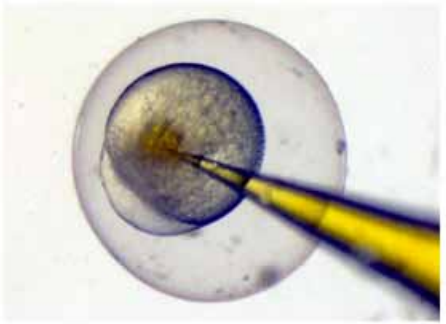


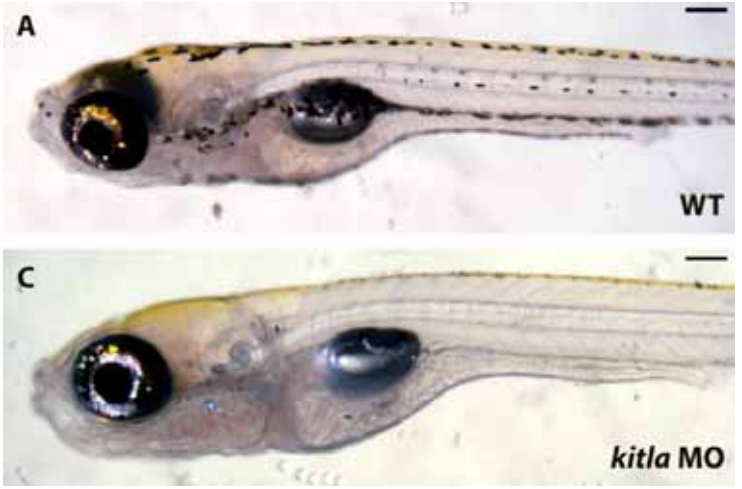
- *In vitro* synthesized 5’ Gppp-capped and polyA-tagged mRNA
- Inject into 1-2 cell stage embryos

c-kit/kit ligand signaling pathway in zebrafish melanocyte development

Gain of function ---
overexpression of kit ligand

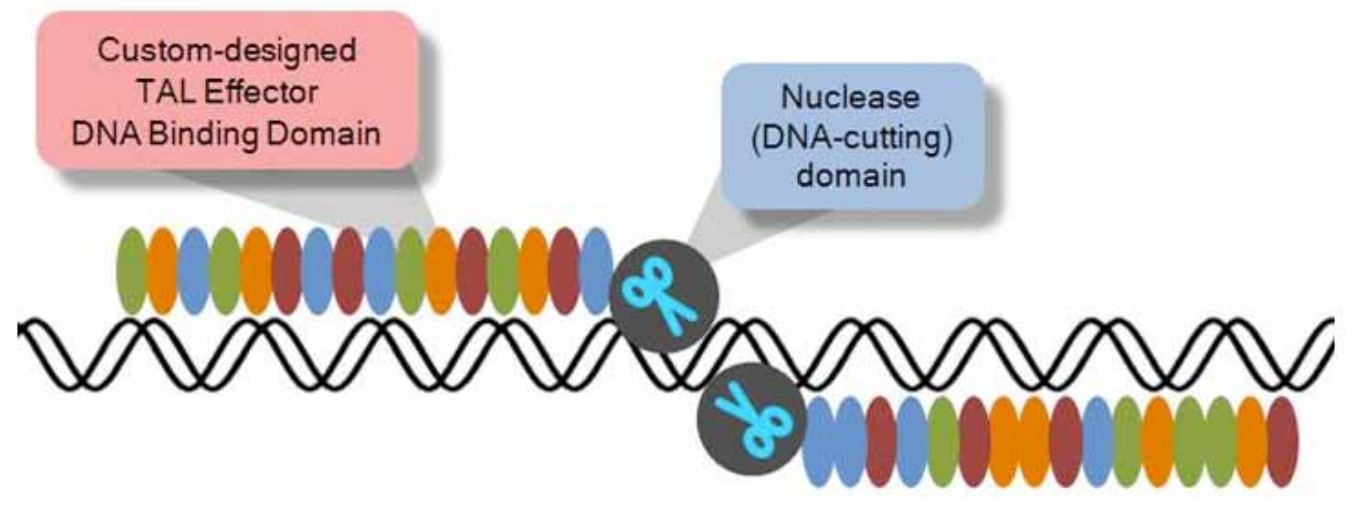
Hyperpigmentation

Loss of function ---
kit ligand morpholino injection



Hypopigmentation

Generation of zebrafish mutants



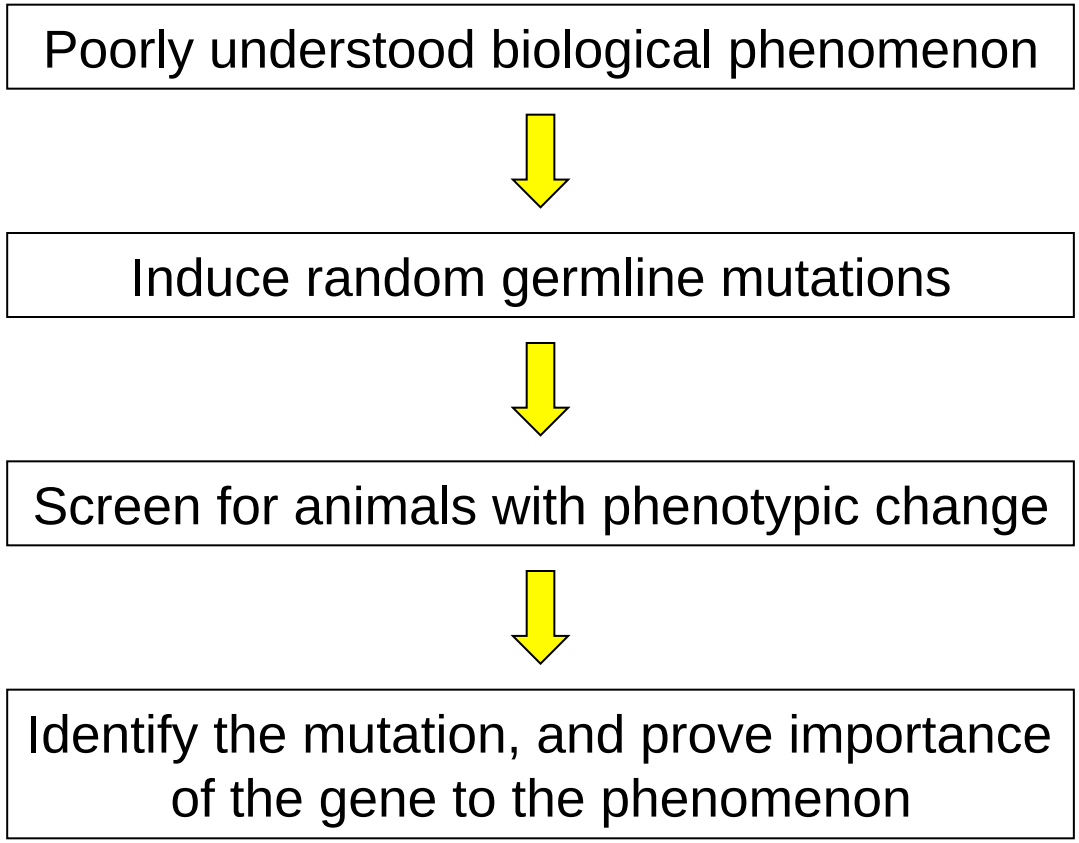
- TAL (transcription activator like) effectors --- transcription activators secreted by plant bacteria *Zanthomonas*
- DNA binding domain of TALEs are composed of an array of modules that can recognize a single DNA base
- Possible to engineer TALEs that specifically bind to a desired DNA sequence
- Engineered TAL effectors are fused to the cleavage domain of FokI nuclease (TALENs)
- TALENs function as a site-specific endonuclease cleaving the target sequence in genome

TALEN-mediated Genome engineering

- Sequence insertion or deletion occurs during the process of repairing the DNA double-strand break
- It results in frame-shift or premature termination of translation products

Forward vs Reverse genetics

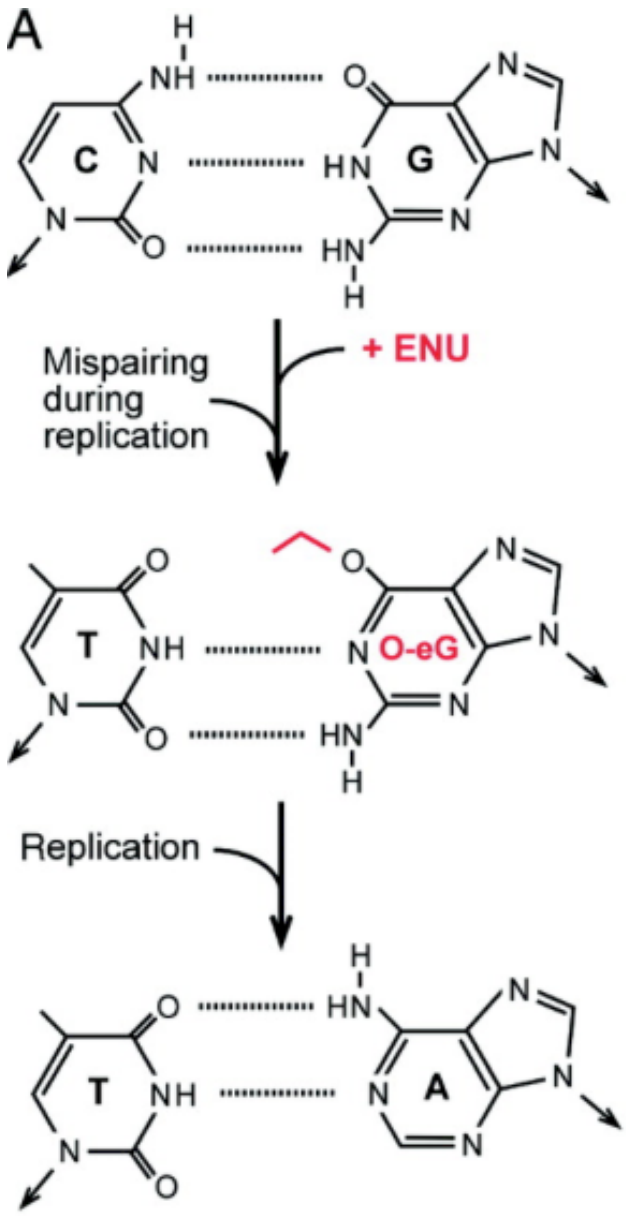
- A way forward to understand protein/gene function



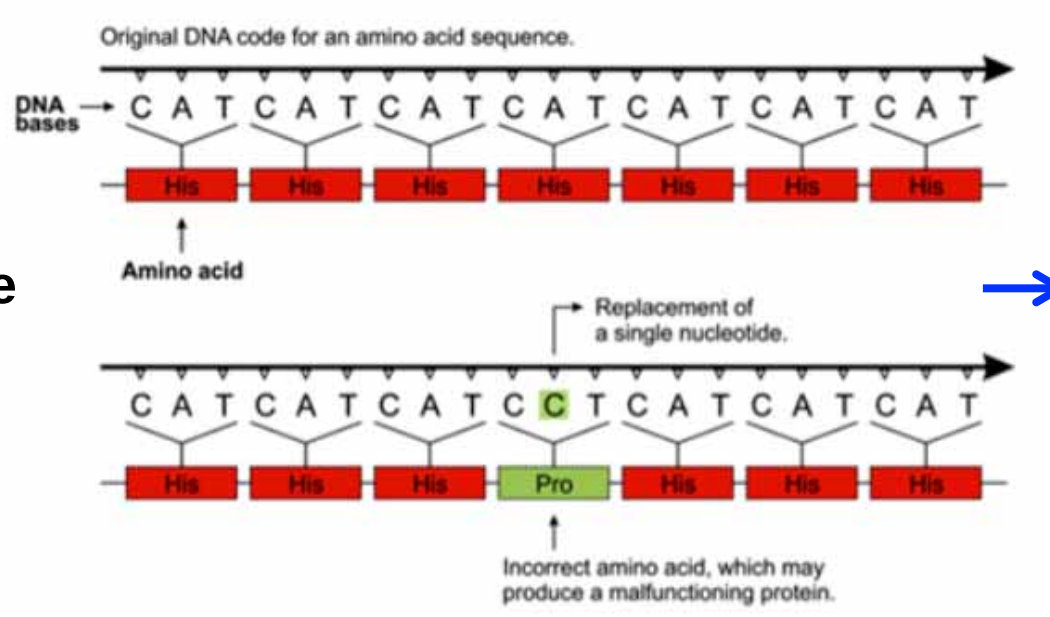
Large-scale ENU mutagenesis screens

- ENU (N-Ethyl-N-Nitrosourea) mutagenesis is a widely accepted and proven method to induce random point mutations

- It induces typically C→T base transversion
  G A



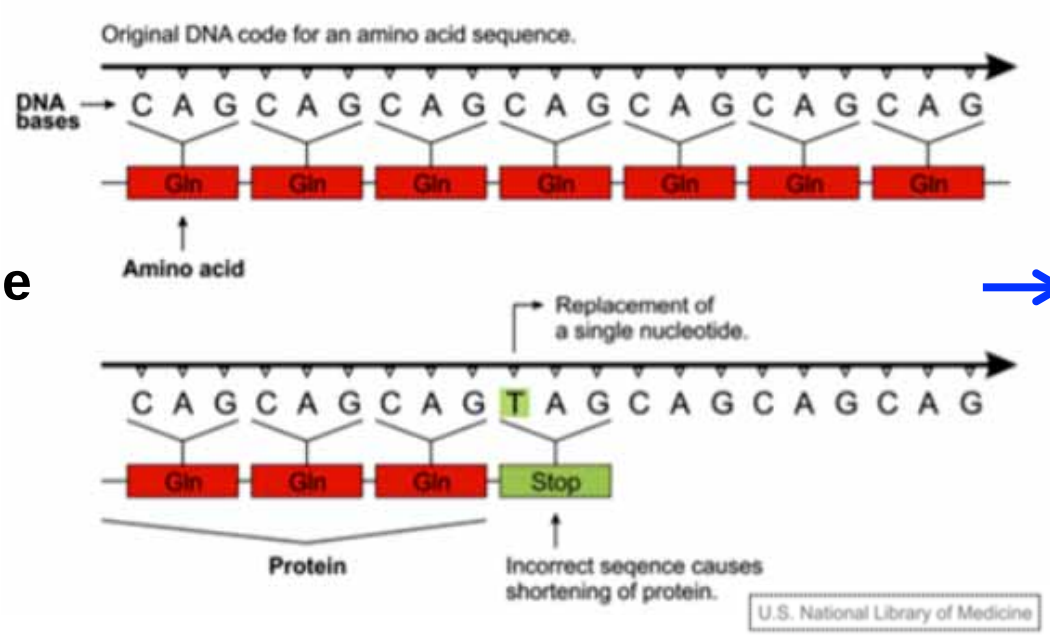
Missense mutation



Substitution of amino acid

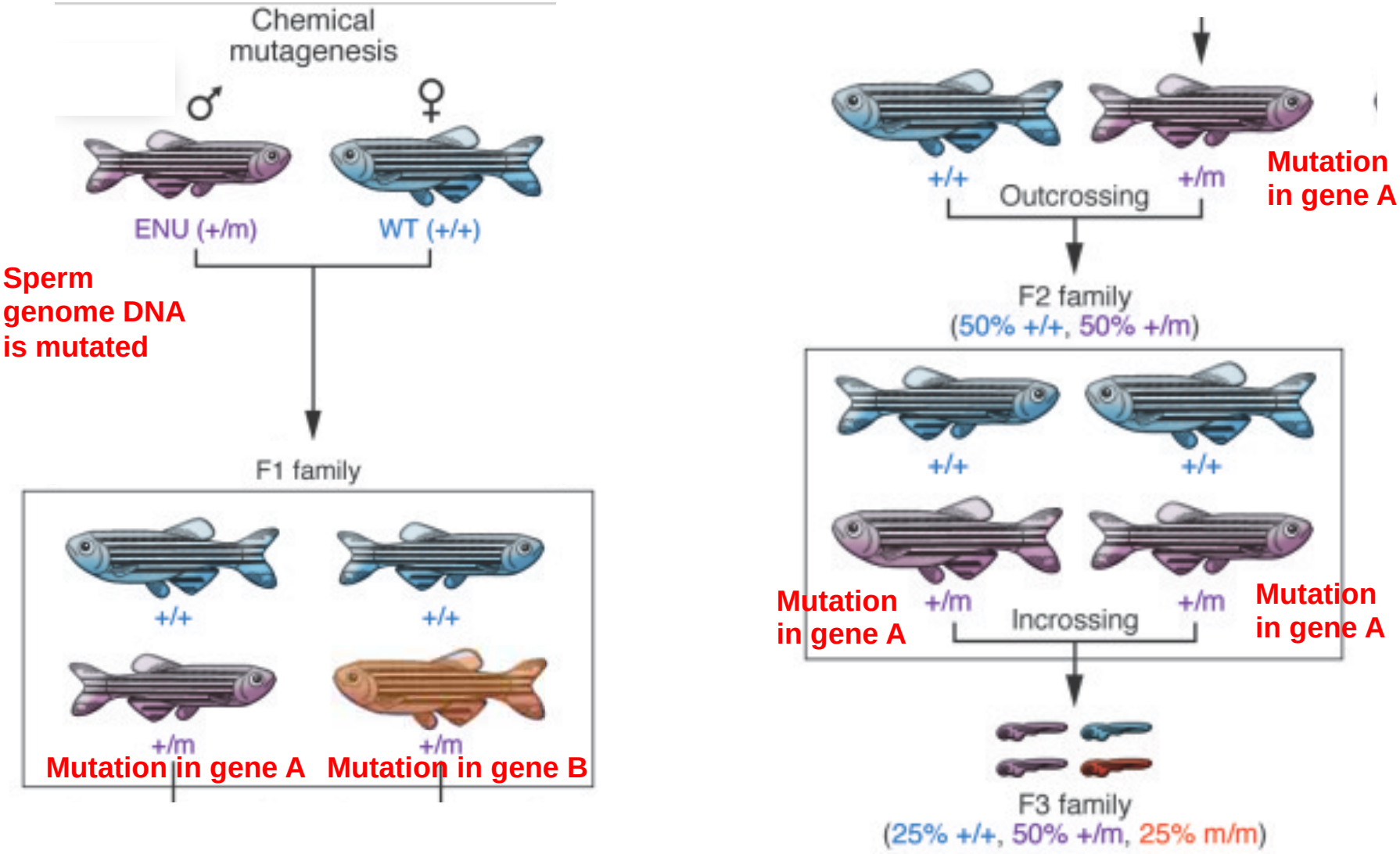
Protein malfunction

Nonsense mutation

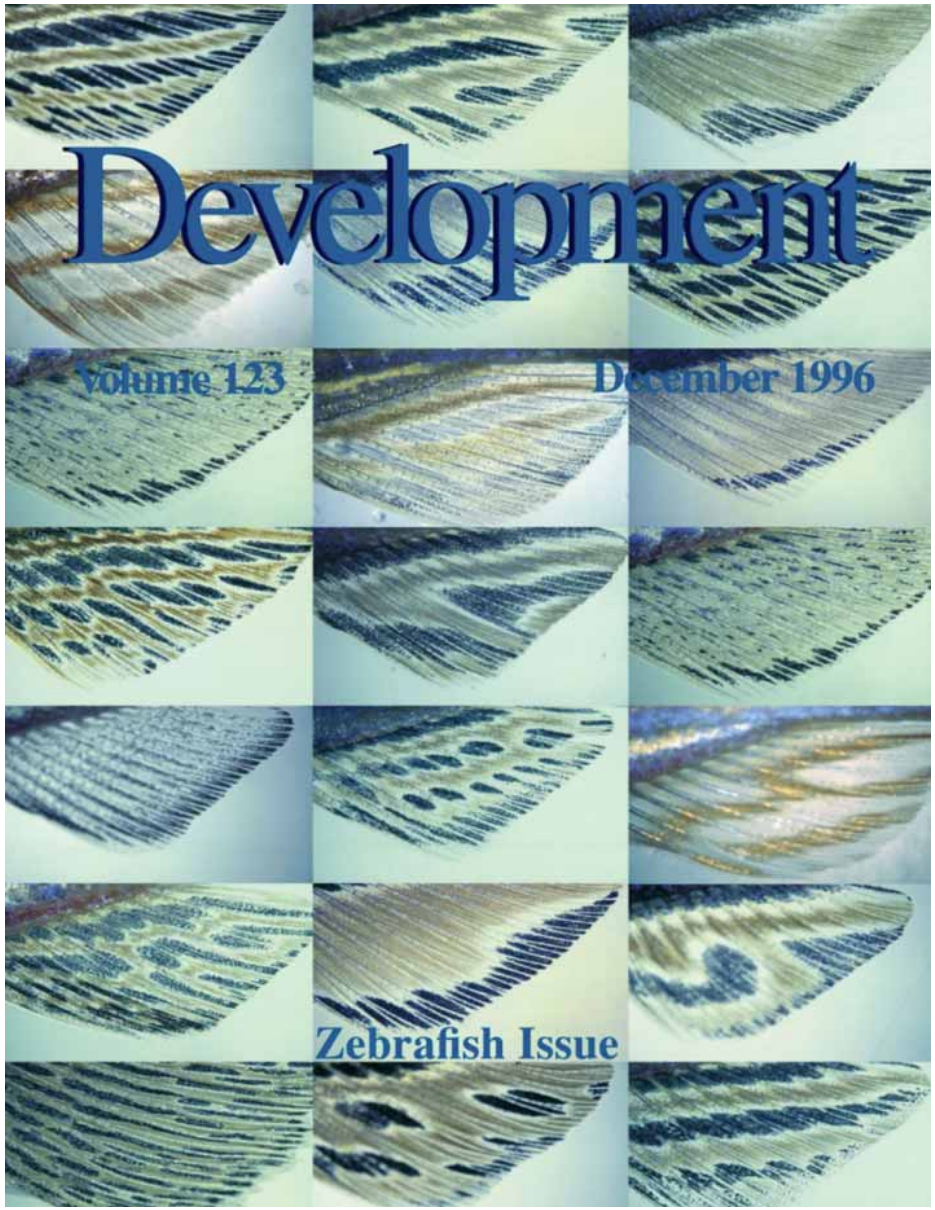


truncation

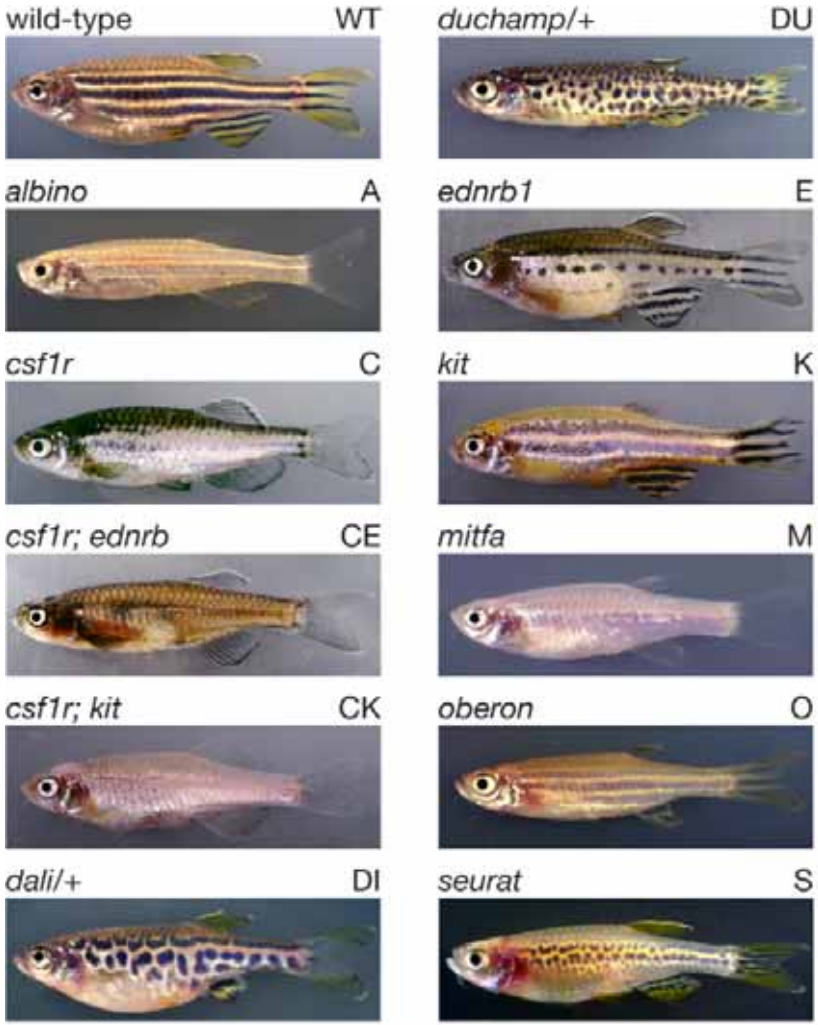
Large-scale ENU mutagenesis screens



Random mutagenesis using ENU is the preferred method in forward genetic approach in zebrafish



Large-scale ENU mutagenesis screens in Boston and Tubingen (1996)

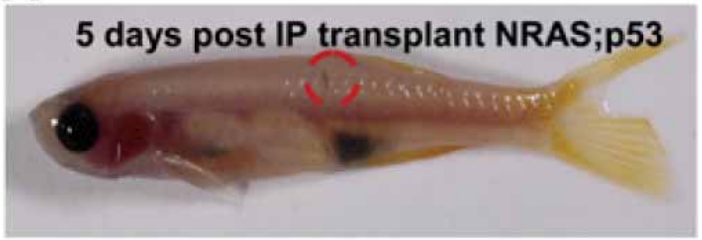


“See-Through” Zebrafish to study metastasis

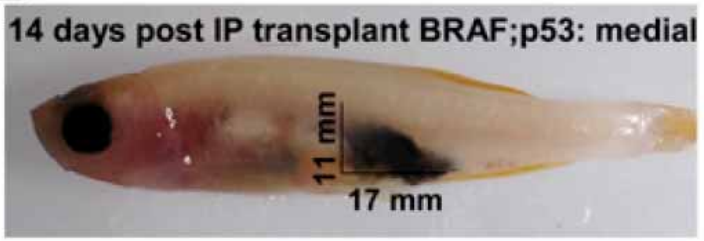
nacre: lack of melanophores (black), mutation in *mitfa*

roy: lack of iridophores (silver), responsible gene is unknown

A



B



White et al., Cell Stem Cell (2007)

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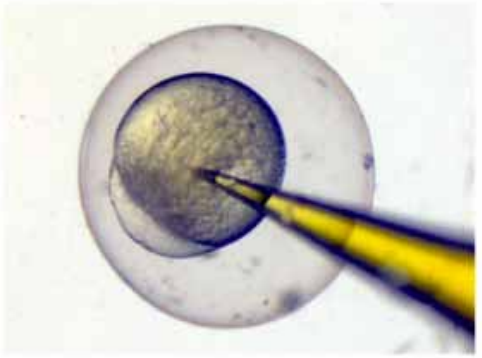

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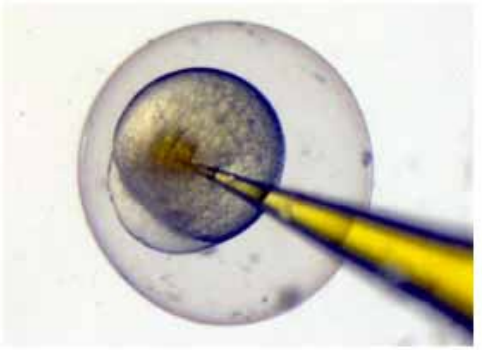
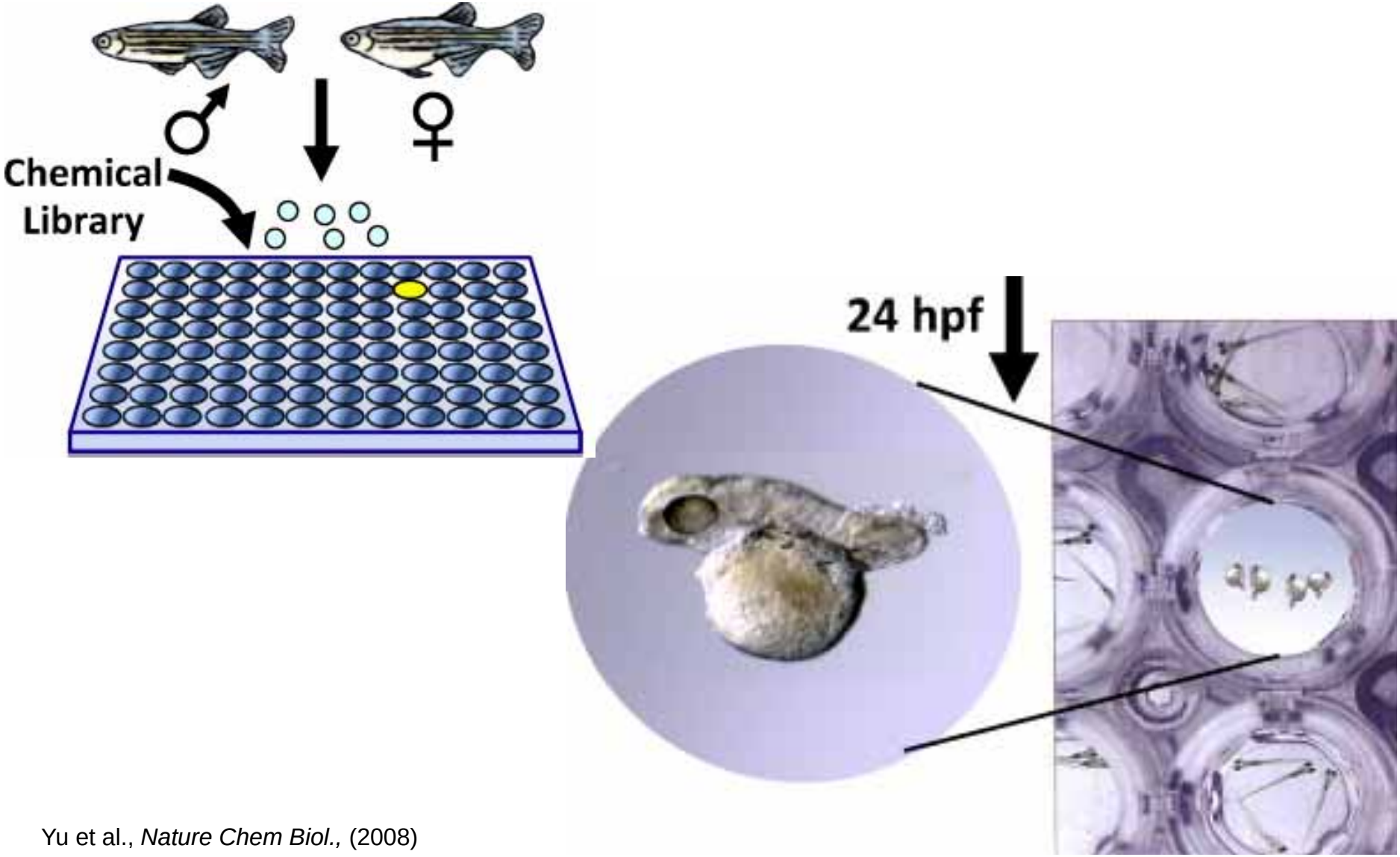


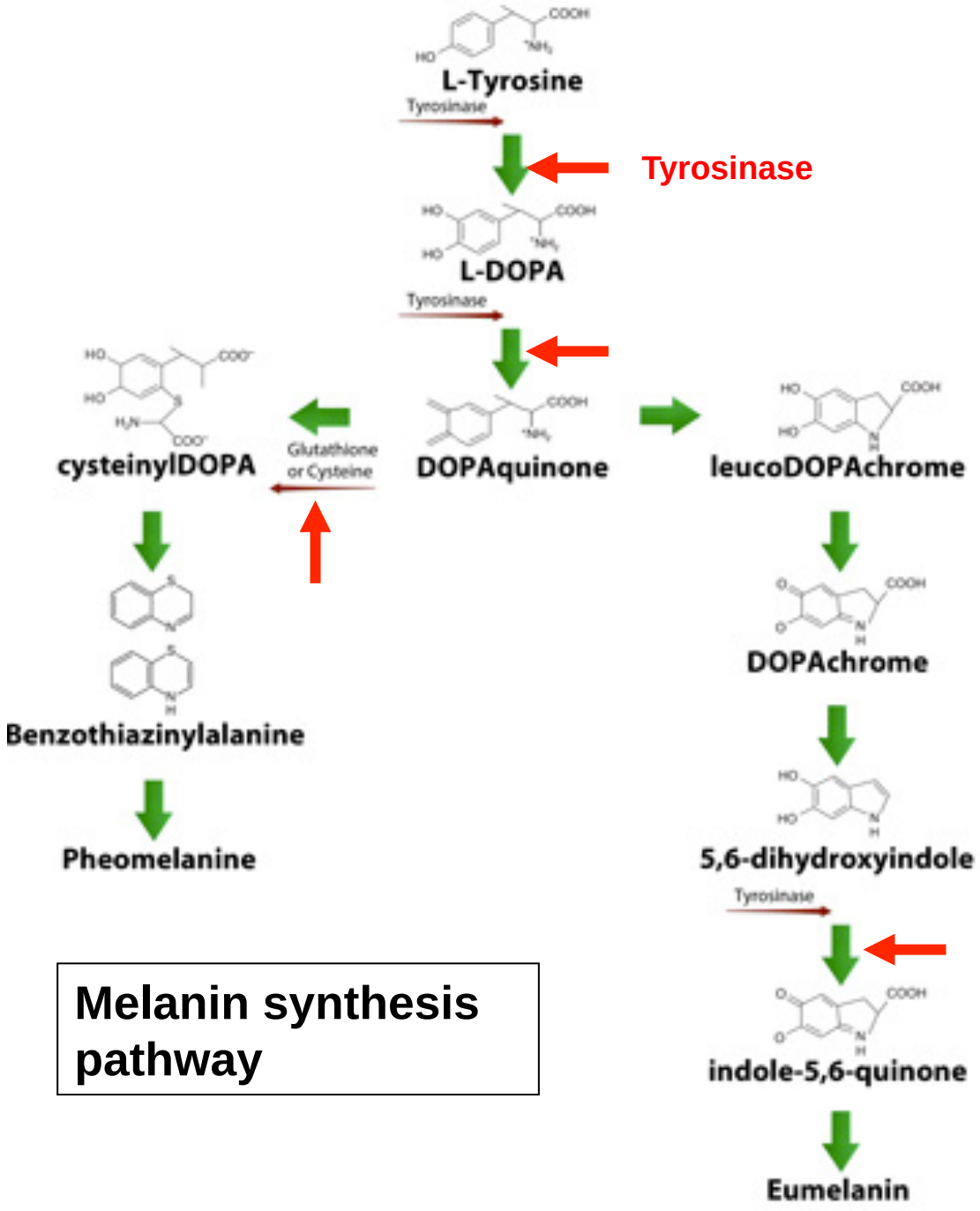
Activate or inhibit protein function by drug treatment



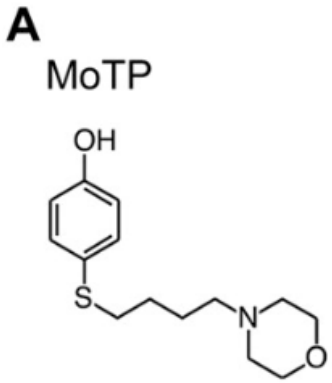
Yu et al., *Nature Chem Biol.,* (2008)

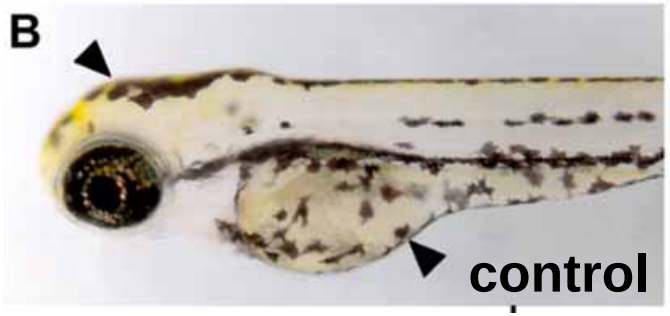
Drug treatment

MoTP --- Inhibitor of Tyrosinase
(melanin synthesizing enzyme)



Melanin synthesis pathway





Objectives

1. To describe zebrafish genetics and technology for biomedical research

2. **To demonstrate the use of zebrafish for biomedical research**

Zebrafish: a model for human disease

Disease, pathological process or exposure	Example of zebrafish model	Phenotype and/or studies of disease pathogenesis	References
Congenital and hereditary disease			
Birth defects, paediatric syndromes	Random mutants from ENU and insertional mutagenesis	Several thousand mutants with early phenotypes affecting developmental processes and organogenesis	16,17,23
Disease-susceptibility traits	ENU-mutation in *adenomatous polyposis coli* (*apc*) gene	*apc*$^{+/-}$ fish develop intestinal polyps	34
	Ataxia telangiectasia morphant	Increased radiation sensitivity	106
Carcinogenesis			
Drug carcinogenicity testing	Chemical carcinogen exposure	Assorted tumours including sarcoma, seminoma	57
Cellular hyperproliferation	Genetic screen for hyperproliferation mutants	*bmyb*$^{-/-}$ ENU mutant with hyperproliferation cell phenotype; a subsequent chemical screen identified a specific suppressor	61,77,105
Oncogenesis	Panel of insertion mutants in ribosomal protein gene loci	Unexpected increased incidence of tumours suggests a new mechanism of oncogenesis	107
Genomic instability	Mutants resulting from forward-genetic screens	Increased incidence of spontaneous tumours or tumour susceptibility	59–61
Leukaemogenesis	Tg(*rag2:Myc*) zebrafish	Lethal acute lymphoblastic leukaemia; conditional variant using Cre-lox technology	63,53
	Tg(*rag2:bcl-2*) zebrafish	Lymphocytosis, transgene conferred steroid- and irradiation-resistance	64
Melanoma oncogenesis	Tg(*mitfa:BRAF*) zebrafish	Malignant melanoma	67
Cooperative tumorigenesis	*p53*$^{-/-}$ ENU mutant zebrafish crossed with tumour-prone zebrafish strains	Accelerated tumorigenesis	45,67
	Interbreeding of transgenic zebrafish expressing leukaemogenic genes	Accelerated leukaemogenesis	66
Infection			
Gram-positive spp.	Infect embryos with *Bacillus subtilis*	Observe leukocyte behaviour	82
Mycobacterium spp.	Infect embryos or adults with *Mycobacterium marinum*	Assess vulnerability, organism virulence, contribution of adaptive cellular immunity, transcriptome response	81,93,96
Gram-negative spp.	Infect embryos with *Escherichia coli*, *Salmonella arizonae*	Observe leukocyte behaviour	82,93
Inflammation and wound healing			
'Sterile' wounding	Transect embryonic tail, or wound fin	Observe, quantitate and modify leukocyte behaviour	83,90–92
Regeneration	Fin transection	Observe and quantitate regrowth	73,108,109
	Removal of cardiac ventricular muscle	Cardiac muscle regeneration	110,111
Immunological disease			
Immune suppression	Immune suppression by irradiation	Myelosuppression; suppresses immune allograft rejection	112,113
	Immune suppression due to T-cell dysfunction	Heightened susceptibility to *M. marinum* infection	96

ENU, ethylnitrosurea; Tg, transgenic model.

Graham et al.,
Nat Rev Genetics
(2007)

Zebrafish: a model for human disease

Disease, pathological process or exposure	Example of zebrafish model	Phenotype and/or studies of disease pathogenesis	References
Metabolic disease			
Iron-storage disorder	ENU-induced mutation in *ferroportin*	Discovery of *ferroportin* basolateral iron transporter, later implicated in Type IV haemochromatosis	114,115
Porphyria (exemplifying inborn errors of metabolism)	ENU-induced mutations affecting various haem-synthesis enzymes	Light-sensitive anaemia and haemolysis	25,26
Endocrine disease			
Hypothyroidism	Expose embryos to anti-thyroid drugs	Biochemical hypothyroidism with developmental effects	116–118
Growth hormone excess	Transgenic overexpression or direct administration	Increase in muscle bulk	119
Nutritional disease			
Fasting and starvation	Restrict food	Affects fin growth	120
Vitamin deficiency	Antagonize vitamin K by warfarin administration	Anti-coagulation	121
Psychological and behavioural abnormalities			
Addiction	Genetic screen for altered cocaine sensitivity	ENU mutants with cocaine insensitivity	39
Social behaviour	Computer-assisted quantification of schooling and chasing behaviour	With tools to quantify behaviour, perturbations can be detected more easily and objectively	35
Mating behaviour	Natural variation in behaviour	Correlate reproductive success with territorial behaviour	122
Cognitive function	Video recording of locomotor activity	Age-related decline in defined cognitive responses, accelerated by genotoxic stress and attenuated by cholinergic upregulation	40
Toxicity and poisoning			
Teratogenicity screening	Transgenic zebrafish with scorable target transgene	Assay mutagenicity of chemicals	123
Teratogenicity mechanisms	Thalidomide treatment of embryos and selected morphants	Antiangiogenic effect is mediated by C2-ceramide and sphingosine-1-phosphate pathway	124
Exposure to environmental chemicals	Arsenate and perchlorate exposure	Disruptive effects on thyroid histology	125

ENU, ethylnitrosurea.

Graham et al., Nat Rev Genetics (2007)

2. Zebrafish: a model for human disease

I. **Carcinogenesis**

T cell lineage acute lymphoblastic leukemia

II. Regeneration study

Heart

III. Personalized medicine

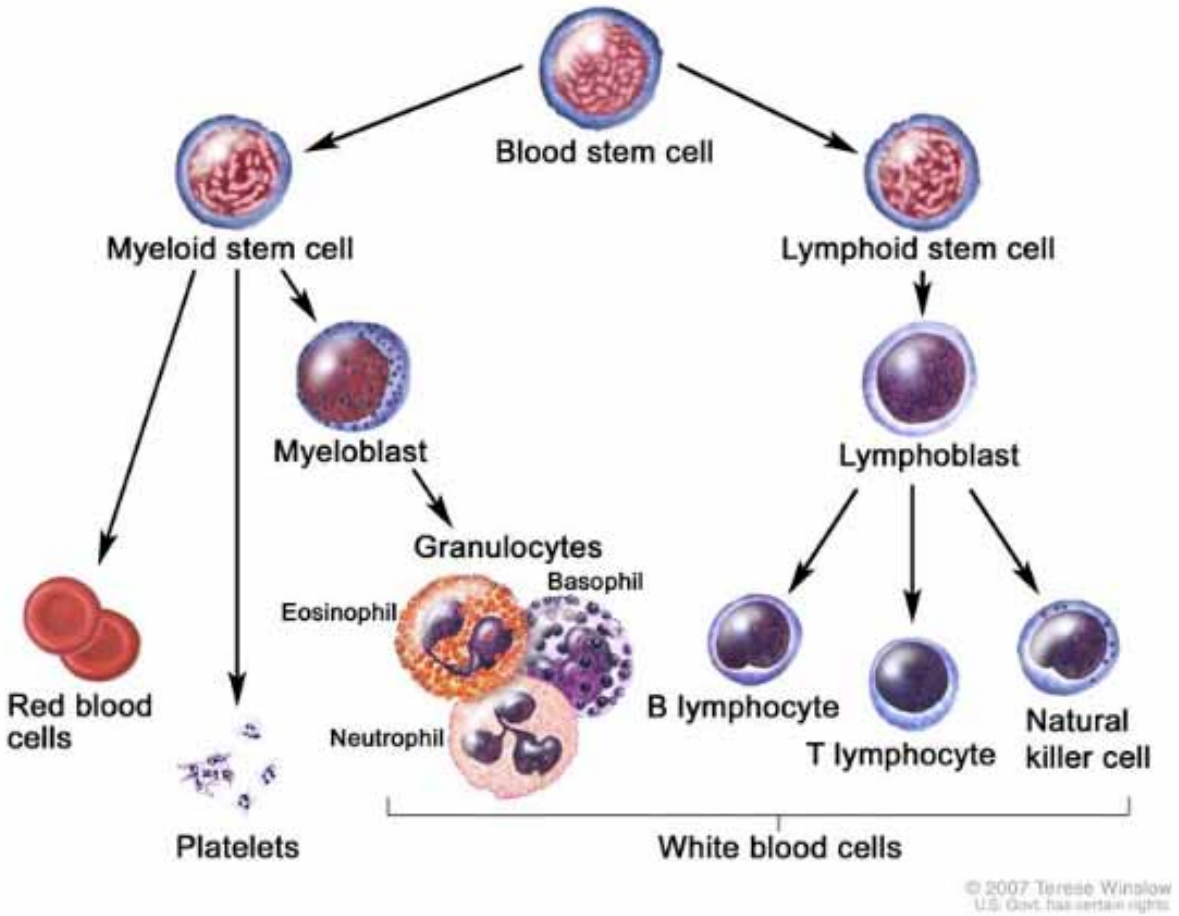
1. Zebrafish carcinogenesis

- Zebrafish develop malignant tumors spontaneously and in response to mutagens
- Could be increased on genetically unstable background (eg. Loss of tumor suppressor genes such as *p53*)

i) Zebrafish T-ALL model

Tg[rag2:mouse-c-Myc]
Tg[rag2:zebrafish-bcl2]
Tg[xenopus-E1a:human-ETV6-Runx1 synonym Tel-AML1]
Tg[rag2:human-Notch1]

T cell lineage acute lymphoblastic leukemia (T-ALL)



- High expression of T-cell oncogenes
- Altered normal mechanisms that control cell proliferation and differentiation during T-lymphocyte development

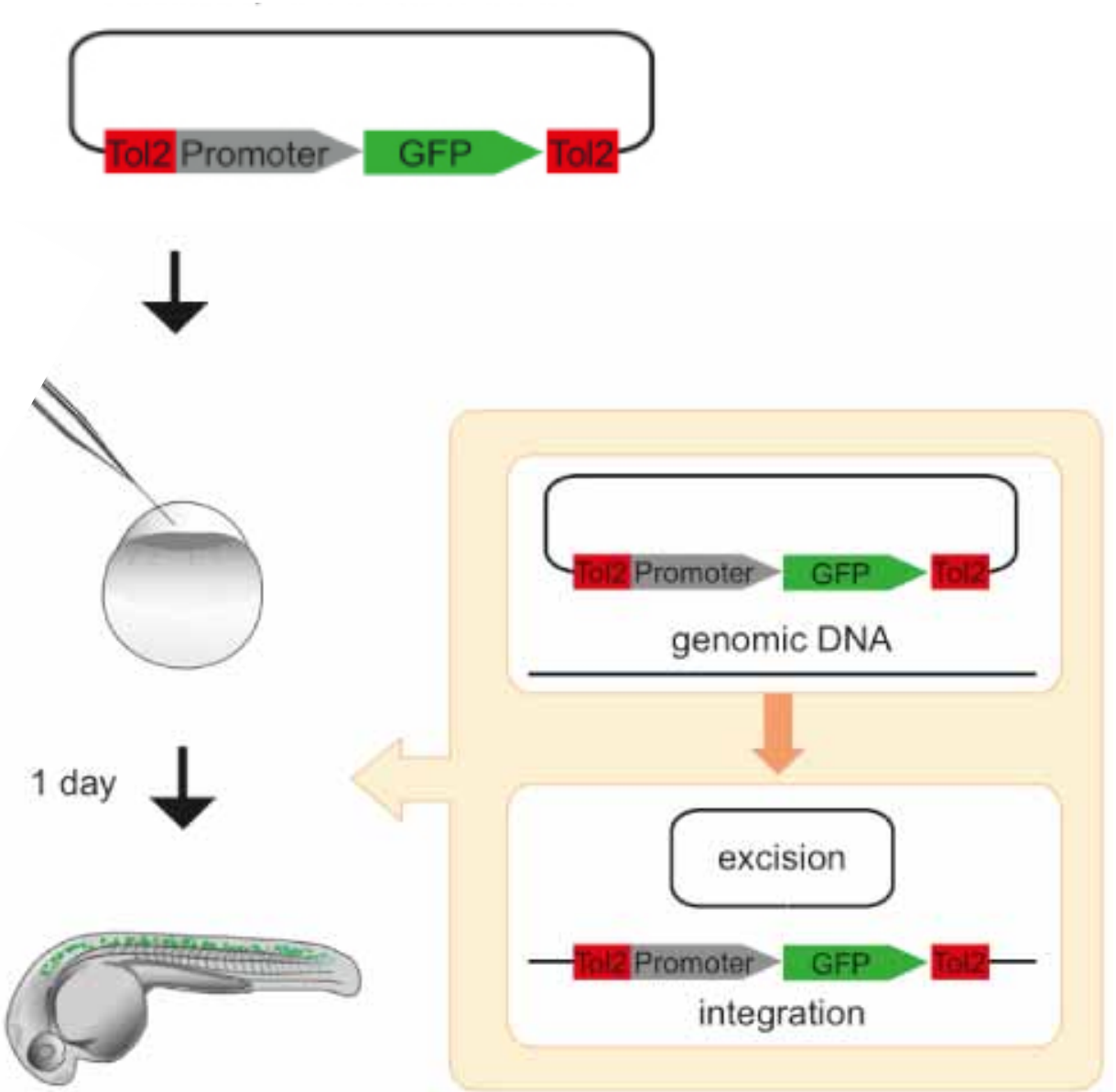
T cell lineage acute lymphoblastic leukemia (T-ALL)

- Aggressive hematologic tumors resulting from malignant transformation of T-cell progenitors
- 10-15% of pediatric and 25% of adult ALL cases
- Chromosomal translocations of T-cell oncogenes

Translocation	Involved gene	Fusion gene function	Frequency
t(7;10)(q34;q24) and t(10;14)(q24;q11)	*TLX1 (HOX11)*	Transcription factor	7% children 31% adults
t(5;14)(q35;q32)	*TLX3 (HOX11L2)*	Transcription factor	20% children 13% adults
inv(7)(p15q34), t(7;7)	*HOXA* genes	Transcription factor	5%
t(1;14)(p32;q11) and t(1;7)(p32;q34)	*TAL1*	Transcription factor	3%
t(7;9)(q34;q32)	*TAL2*	Transcription factor	<1%
t(7;19)(q34;p13)	*LYL1*	Transcription factor	<1%
t(14;21)(q11.2;q22)	*BHLHB1*	Transcription factor	<1%
t(11;14)(p15;q11)	*LMO1*	Protein-protein interaction	2%
t(11;14)(p13;q11) and t(7;11)(q35;p13)	*LMO2*	Protein-protein interaction	3%
t(1;7)(p34;q34)	*LCK*	Signal transduction	<1%
t (7;9)(q34;q34.3)	*NOTCH1*	Fate determination, differentiation	<1%
t(7;12)(q34;p13) and t(12;14)(p13;q11)	*CCND2*	Cell cycle activator	<1%

Haematologica. 2009 February; 94(2)

Generation of transgenic zebrafish

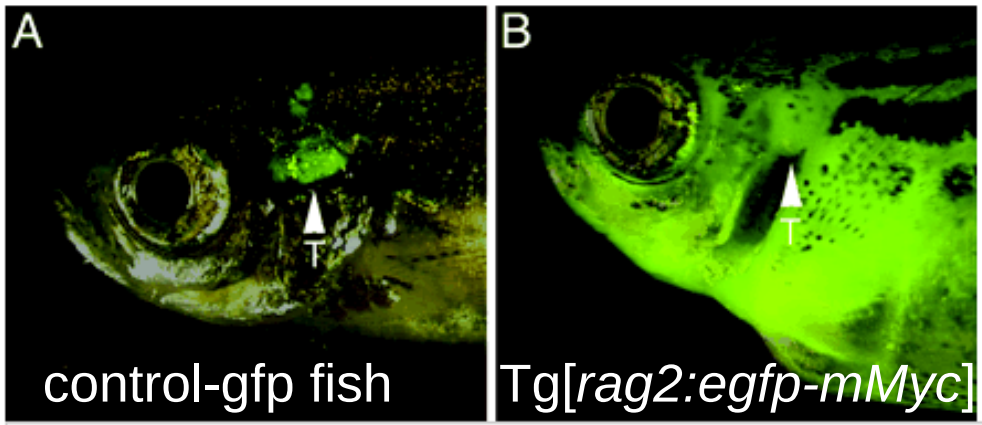


- Insert cDNAs encoding proteins of interest downstream of promoter (ubiquitous, inducible or tissue-specific)
- Inject 1-cell stage embryos with tol2 transposase mRNA
- DNA will be integrated to the genomic DNA
- Raise them and screen the germ cell integration

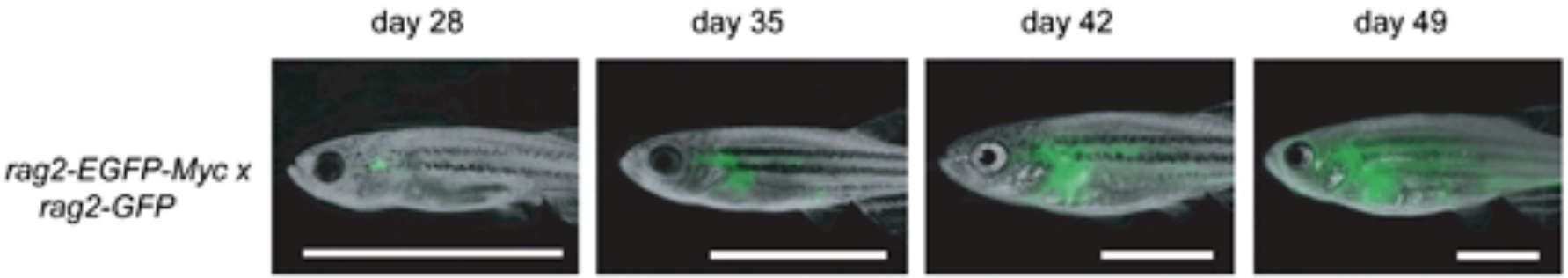
Zebrafish T-ALL model, Tg[*rag2*:*c-Myc*]

Rag2:

- V(D)J recombination-activation protein 2
- Required for chromosomal rearrangement of T-cell antigen receptors



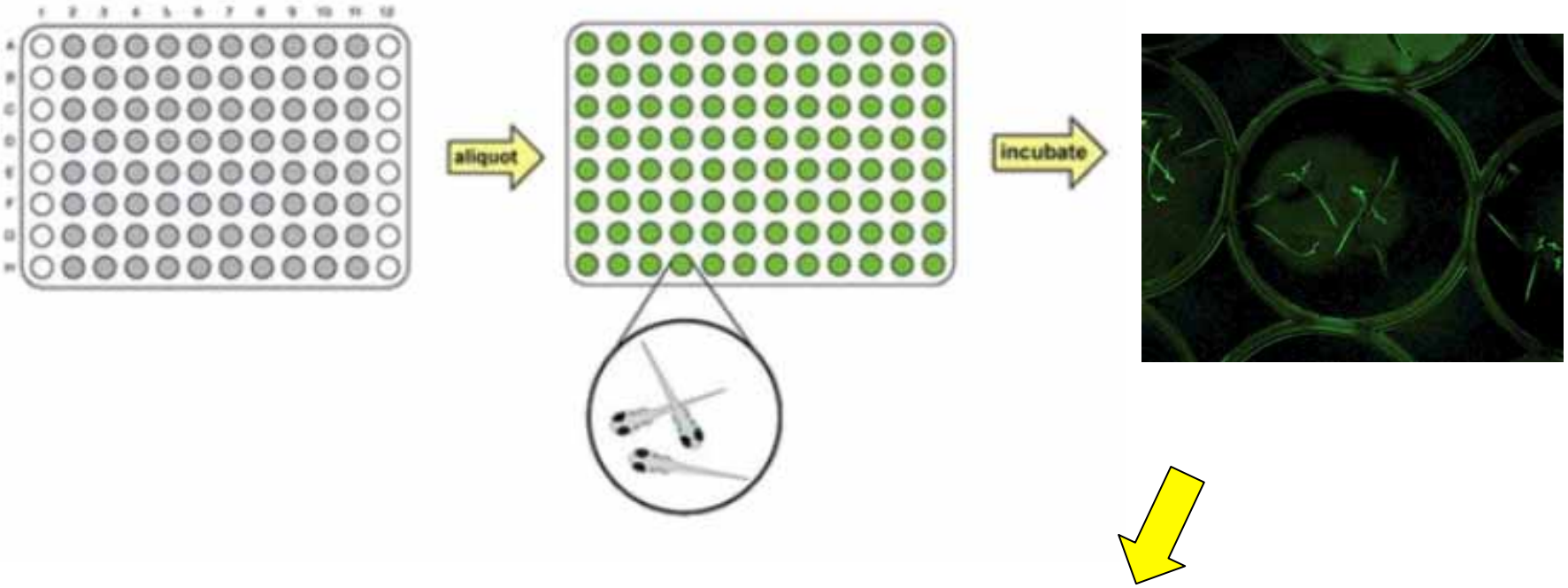
Langenau et al., PNAS (2005)



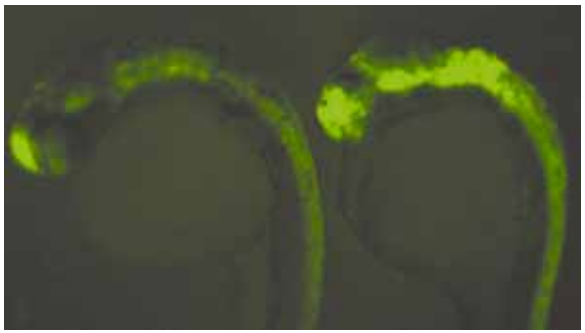
Chemical screens using T-ALL zebrafish

Pooled chemical library

Rag2:myc-egf zebrafish larvae



Compound A
– weak GFP signal

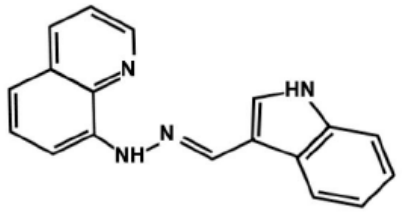


Compound B
– strong GFP signal

blood

2012 119: 5621-5631
Prepublished online April 9, 2012;
doi:10.1182/blood-2011-12-398818

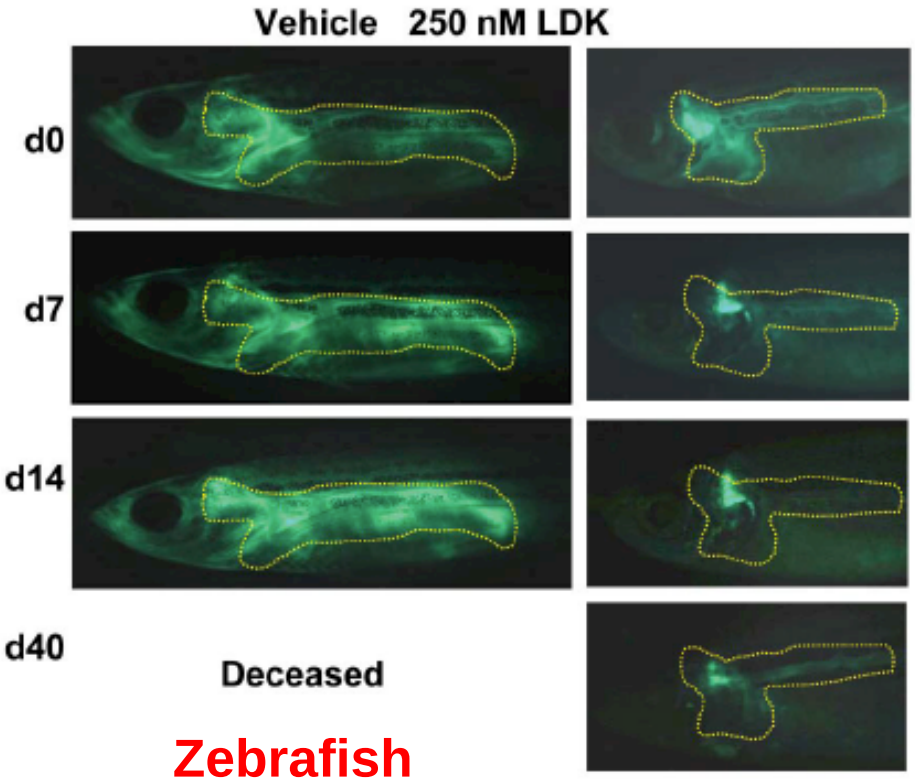
$C_{18}H_{14}N_4$
mw = 286.3



LDK

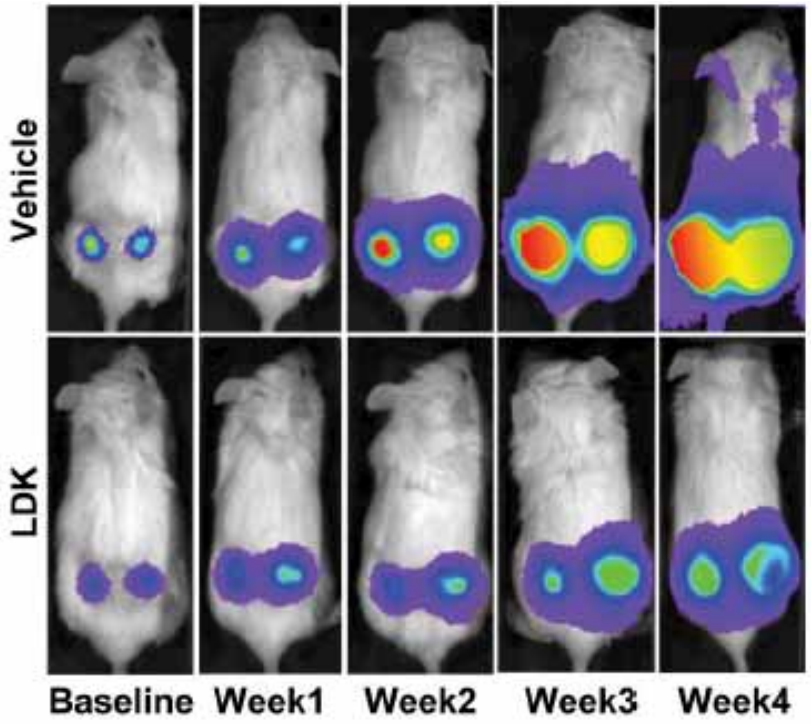
Zebrafish screen identifies novel compound with selective toxicity against leukemia

Suzanne Ridges, Will L. Heaton, Deepa Joshi, Henry Choi, Anna Eiring, Lance Batchelor, Priya Choudhry, Elizabeth J. Manos, Hossein Sofla, Ali Sanati, Seth Welborn, Archana Agarwal, Gerald J. Spangrude, Rodney R. Miles, James E. Cox, J. Kimble Frazer, Michael Deininger, Kaveri Balan, Matthew Sigman, Markus Müschen, Tatiana Perova, Radia Johnson, Bertrand Montpellier, Cynthia J. Guidos, David A. Jones and Nikolaus S. Trede



Zebrafish

Mice



2. Zebrafish: a model for human disease

I. Carcinogenesis

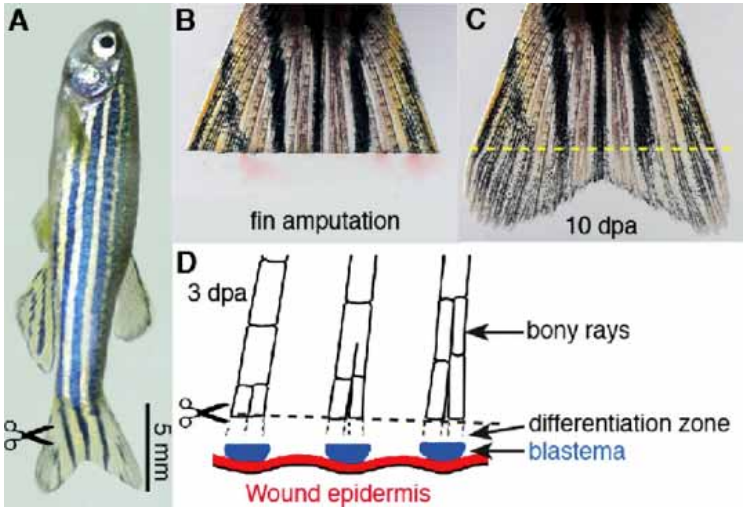
T cell lineage acute lymphoblastic leukemia

II. Regeneration study

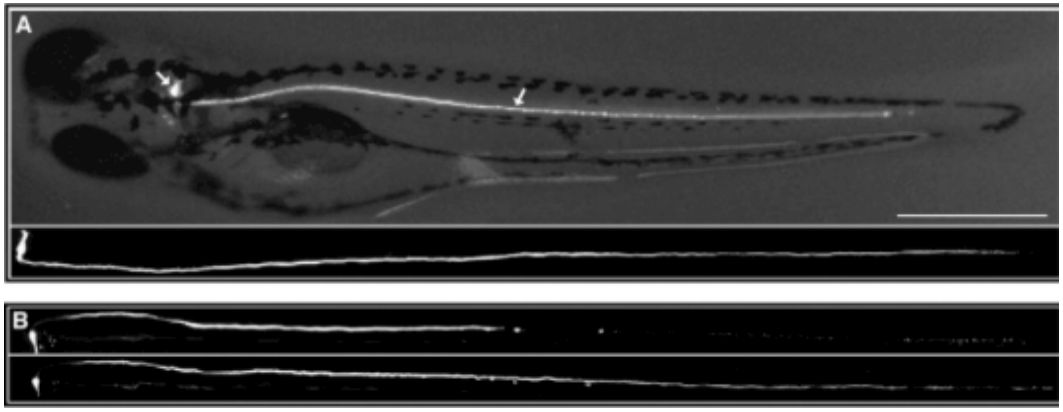
Heart

III. Personalized medicine

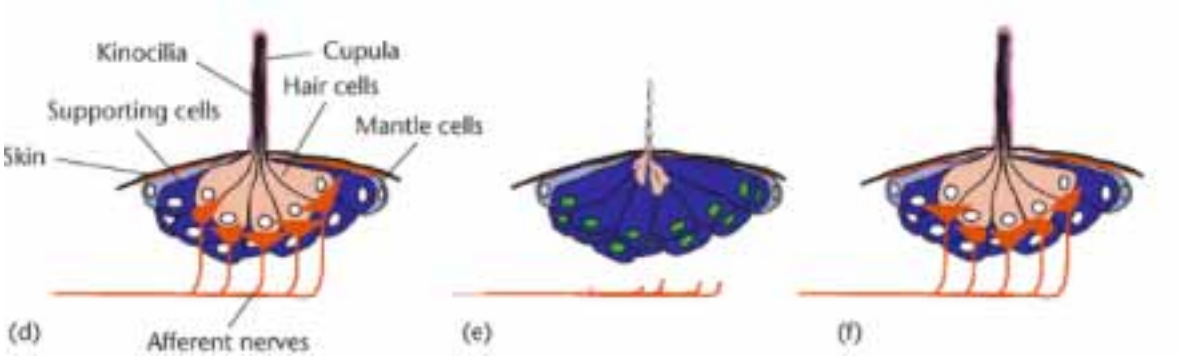
2. Regeneration model using zebrafish



1. Fin



2. Spinal nerve



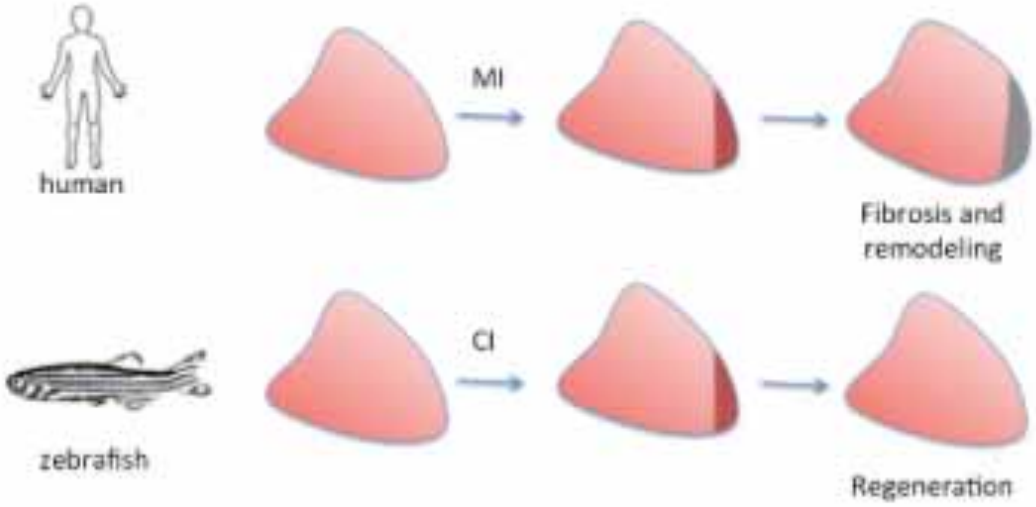
3. Sensory hair cells

How and why does tissue regeneration occur in fish but not in human?

The answers will have the potential to develop therapeutic protocols to cure organ damages in human

Heart failure and heart regeneration

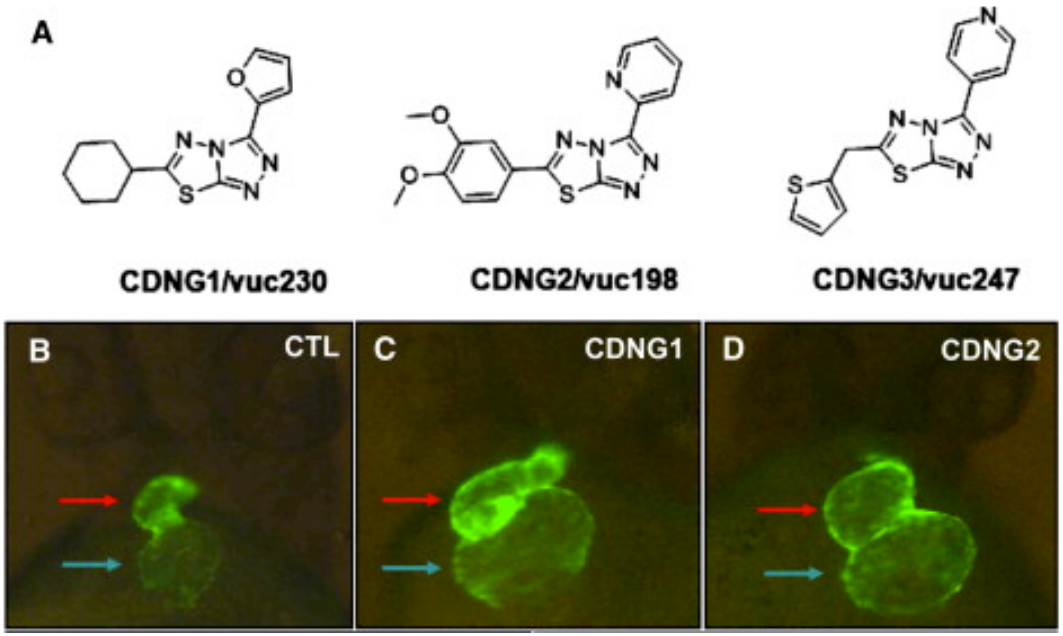
- Heart failure is one of the key causes of morbidity and mortality around the world
- The underlying cause is the loss of functional myocardium
- Mammalian hearts do not regenerate
- Available medical therapies CAN NOT reverse the loss of functional myocardium



- The only available cure is heart transplantation
- On the other hand, zebrafish heart regenerate!!

Identification of small molecules that promote cardiomyocyte proliferation

Cardionogen (CDNG)-1,-2,-3



Heart @48 hpf

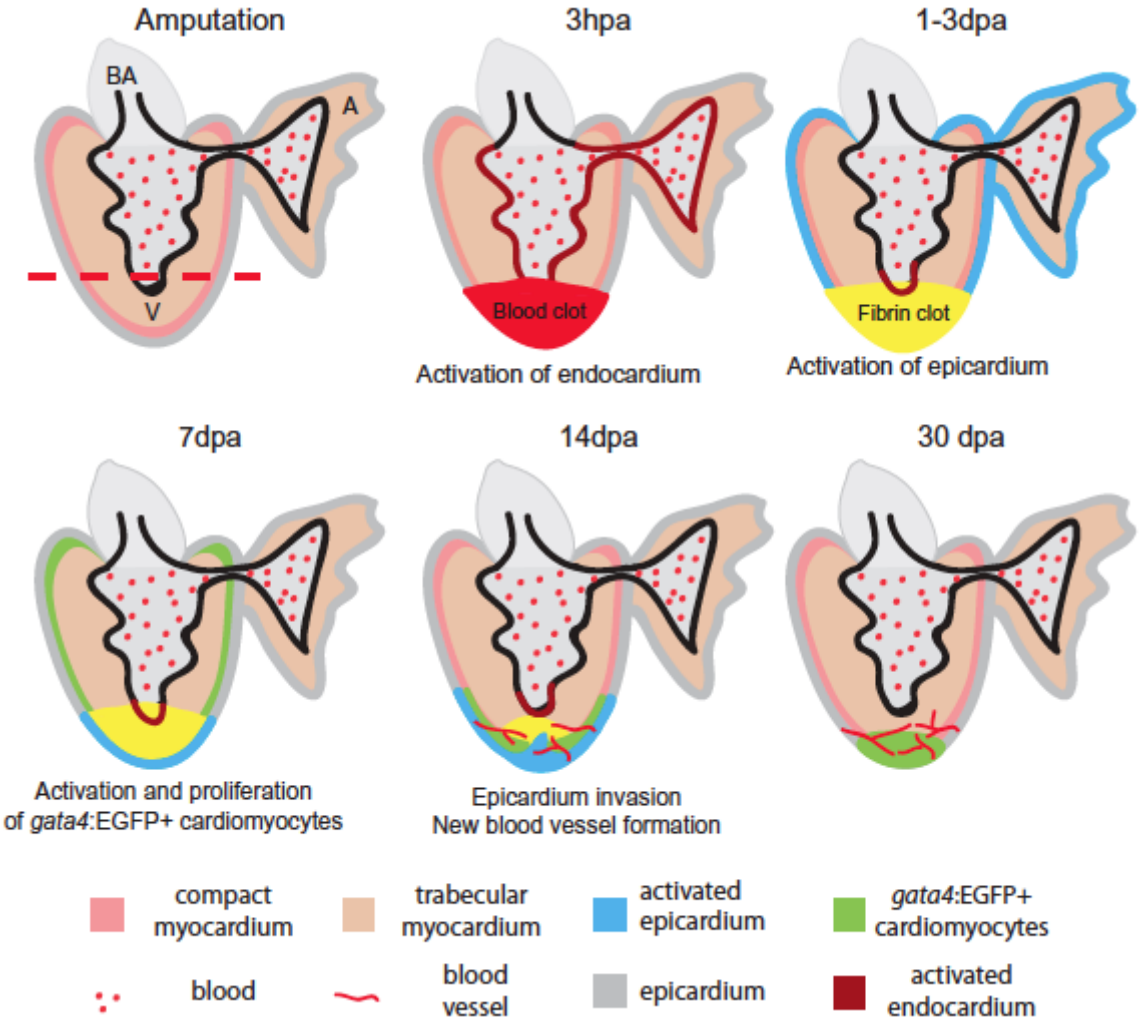
Heidi et al., *Plos Biol.,* (2007)

Terri et al., *Chemistry & Biology,* (2011)

13 DECEMBER 2002 VOL 298 SCIENCE

Heart Regeneration in Zebrafish

Kenneth D. Poss,* Lindsay G. Wilson, Mark T. Keating*

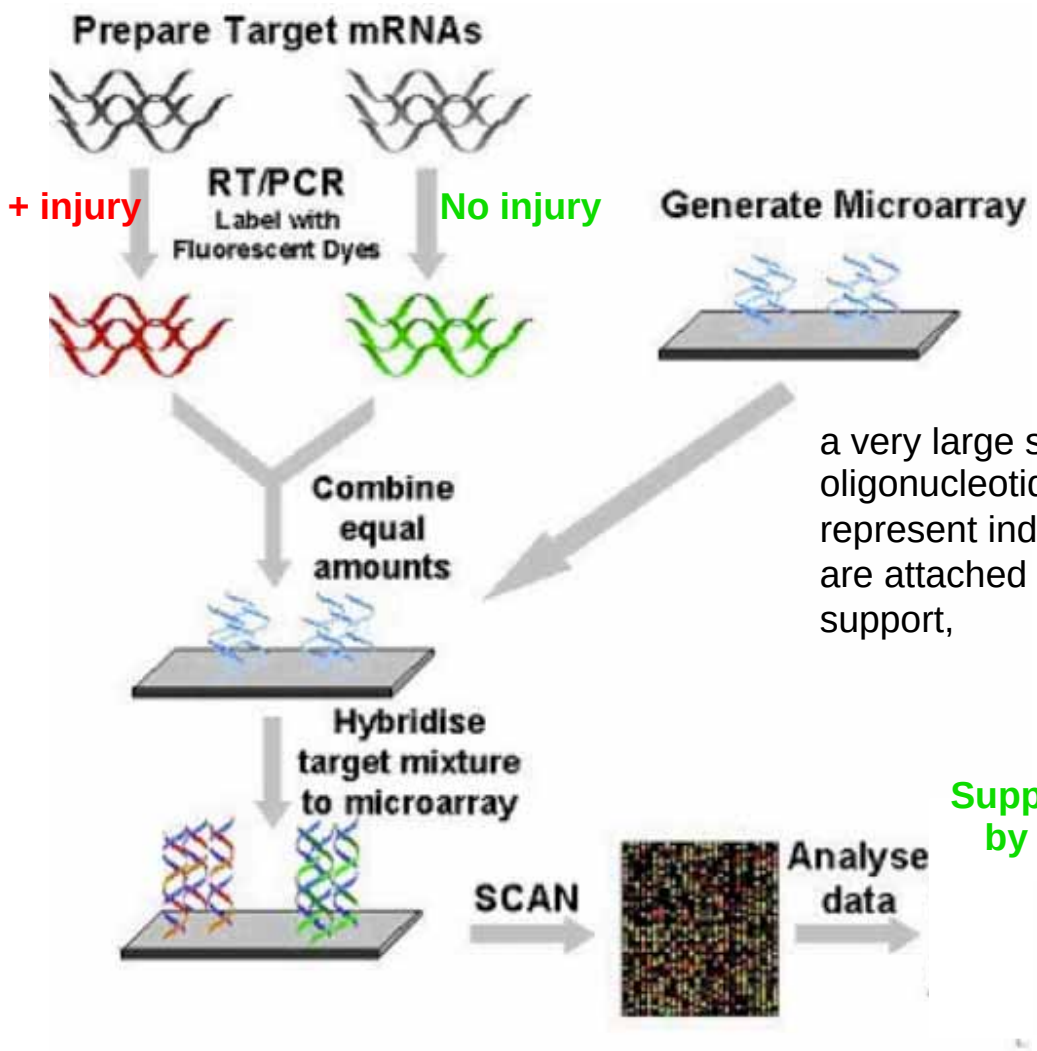


Elucidating the molecular mechanism of zebrafish heart regeneration may provide insight into potential therapeutic approaches for human heart injury

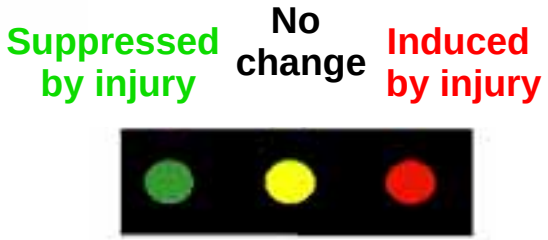
http://www.hhmi.org/biointeractive/stemcells/zebrafish_regen.html

The mechanism of zebrafish heart regeneration

Analyze changes in gene expression during heart regeneration



a very large set of oligonucleotides that represent individual genes are attached to a solid support,



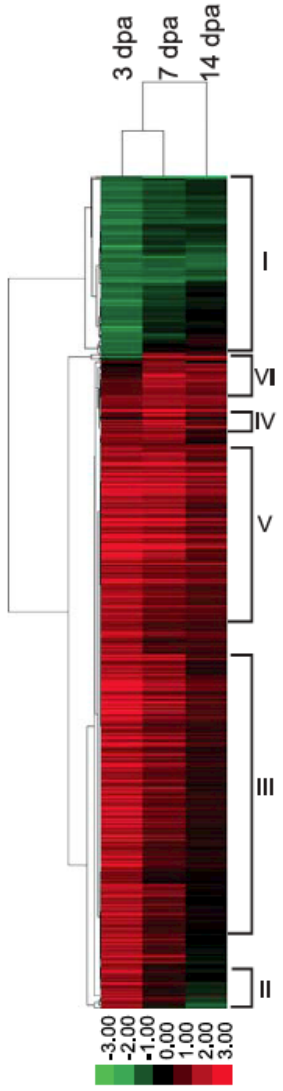
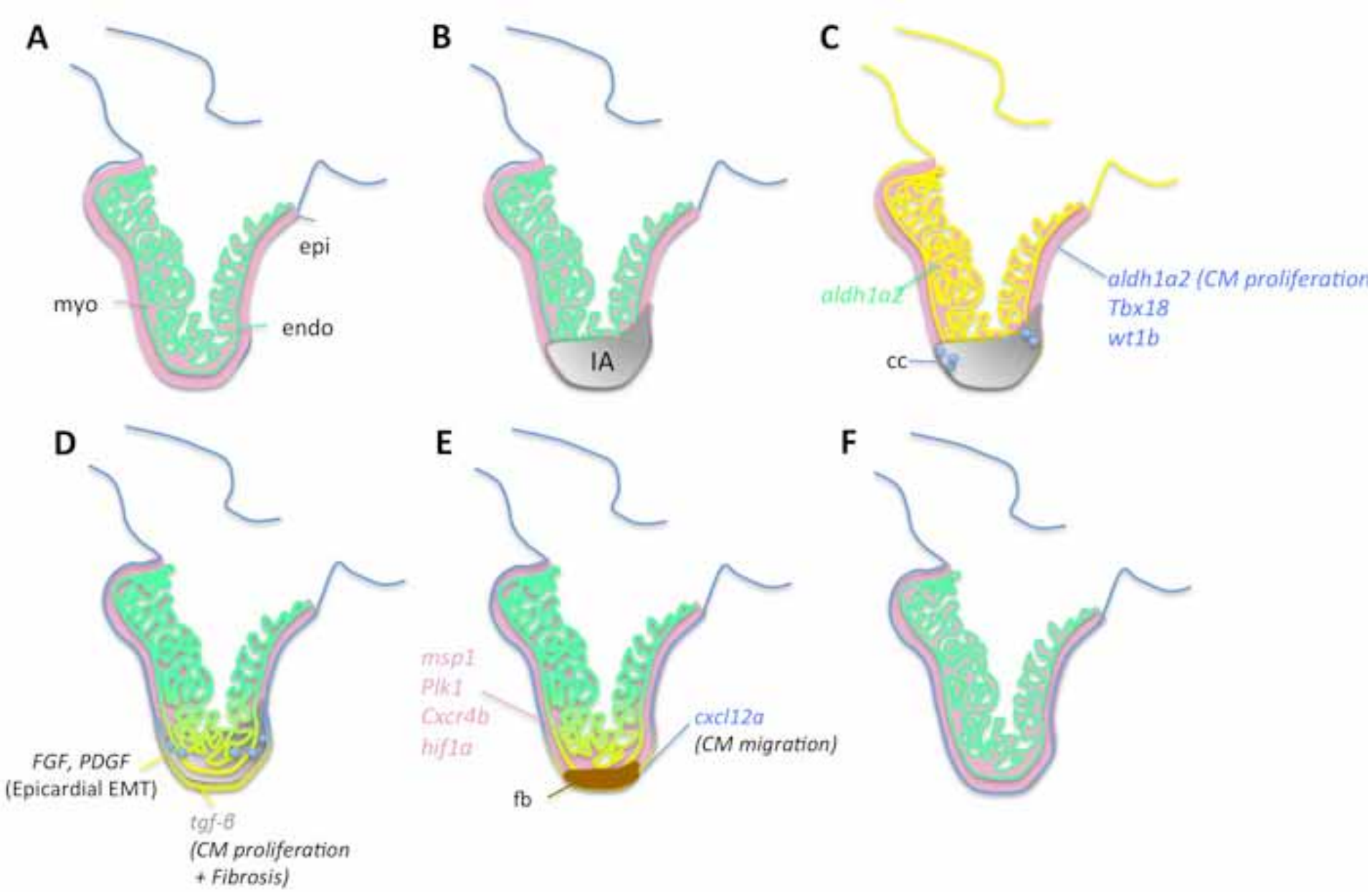


Figure 1. Gene Expression Profili

Factors that regulate heart regeneration



2. Zebrafish: a model for human disease

I. Carcinogenesis

T cell lineage acute lymphoblastic leukemia

II. Regeneration study

Heart

III. Personalized medicine

Functional Assessment of Human Coding Mutations Affecting Skin Pigmentation Using Zebrafish

Zurab R. Tsetskhladze[1,6]*, Victor A. Canfield[2], Khai C. Ang[1,6], Steven M. Wentzel[1,6], Katherine P. Reid[1,6], Arthur S. Berg[3], Stephen L. Johnson[4], Koichi Kawakami[5], Keith C. Cheng[1,6]*

PLOS ONE October 2012 | Volume 7 | Issue 10 | e47398

HuZOR

"Humanized Zebrafish Orthologous Rescue"

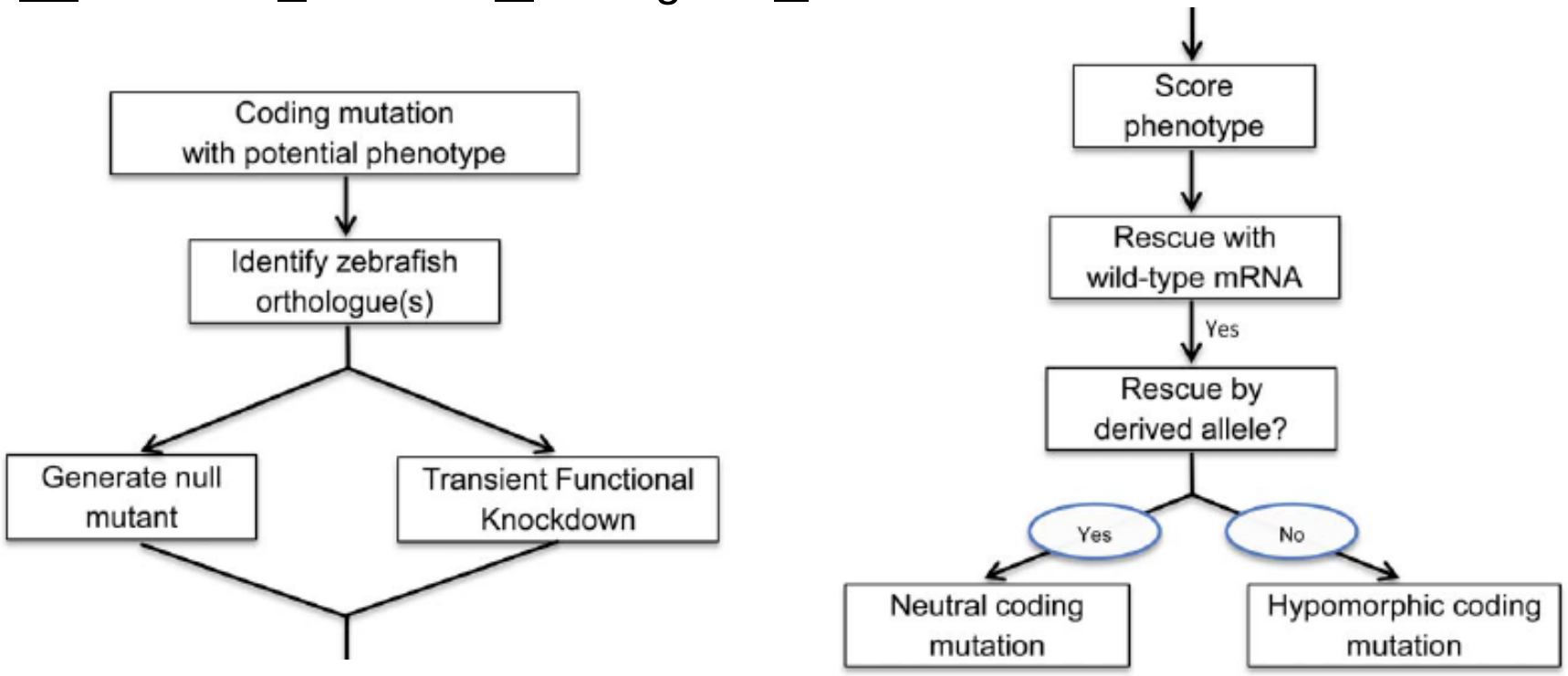


Figure 6. Flow chart for testing coding mutations based on the HuZOR approach. Candidate functional coding mutations are first

A major challenge of personalized medicine

- Personalized medication – the use of genomic information to customize health management
- Every human has several thousand sequence variants that alter coding sequences
- It aims to help select the proper medication and dosages
- It might be used to assess a patient's risk factor for a number of diseases

- There is a need for *in vivo* experimental approaches to assess the functional significance of individual mutations

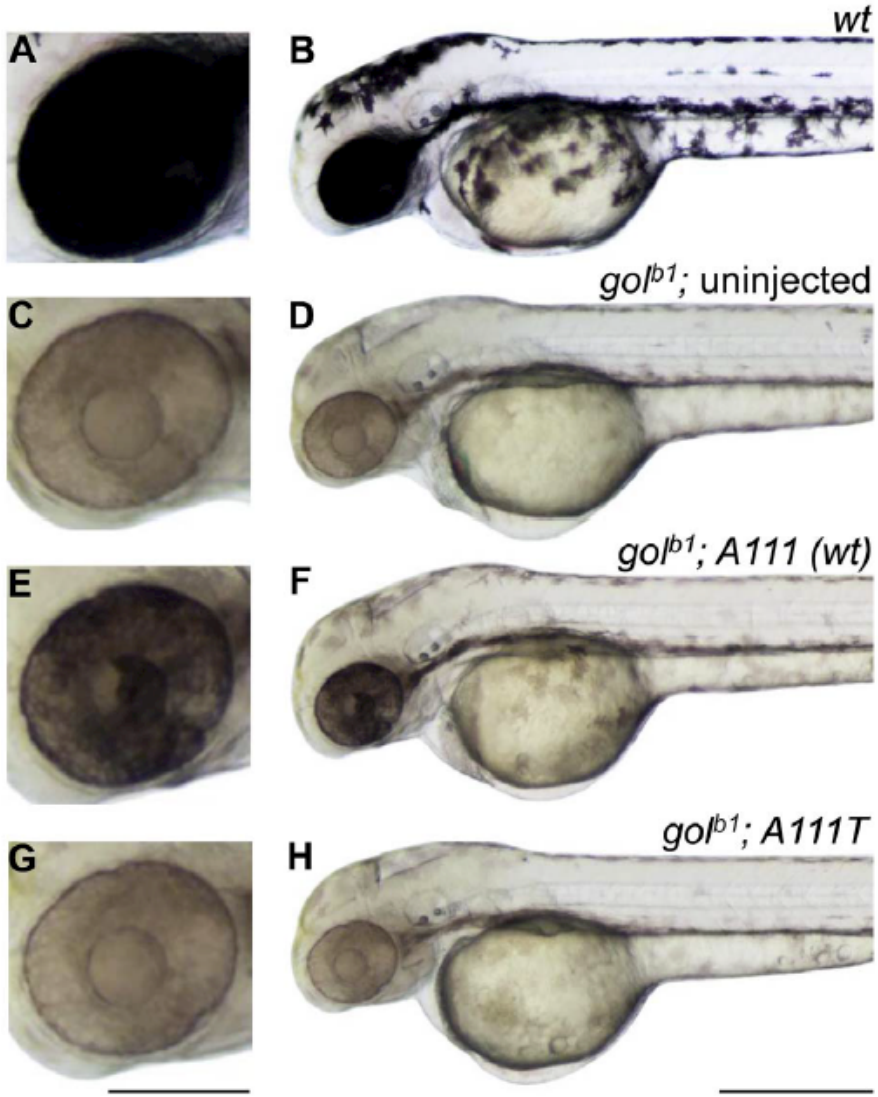


Figure 4. Effect of a human coding polymorphism on zebrafish mRNA rescue of the *golden* phenotype. Lateral views of 48-hpf (A and B) *wt* zebrafish larva (C and D) *gol*^{b1} zebrafish larva (E and F) *gol*^{b1} larva injected with full-length zebrafish *slc24a5 (wt) mRNA* (500 pg) and (G and H) *gol*^{b1} larva injected with full-length zebrafish *slc24a5 mRNA* with a single nucleotide change (500 pg), coding for the orthologous human derived *A111T* allele. Scale bars in (A, C, E, G) 150 μm, (B, D, F, H) 400 μm.
doi:10.1371/journal.pone.0047398.g004

Hypomorphic polymorphism SLC24A5 (A111T) is linked to lighter skin color in Europeans

Slc24a5(A111T) mRNA injection failed to rescue pigmentation defects

Na/K/Ca exchanger

The zebrafish model offers a major opportunity to discover important pathways underlying human disease and to identify novel therapies in high-throughput drug screens, in a way that mice never could…

http://www.devbio.biology.gatech.edu/?page_id=6534

Miho Matsuda, Ph.D.
Assistant Professor
Rutgers, The State University of New Jersey
RBHS, New Jersey Medical School
Department of Cell Biology and Molecular Medicine
185 S Orange Ave, MSB G-671, I-720
Newark, NJ 07103
matsudmi@njms.rutgers.edu
973-972-8926 (office)
973-972-8925 (lab)