Children's Hospital Boston
Vascular Biology Program
Research Associate

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Feasibility of Multiplex CRISPR-mediated Germline Modification

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Selwyn P. Oskowitz Lecture

- CRISPR Background
- Using CRISPR as a Tool in Genetics
- Potential Utility and Current Limitations of Genome Editing

Repurposing Bacterial Immune Systems

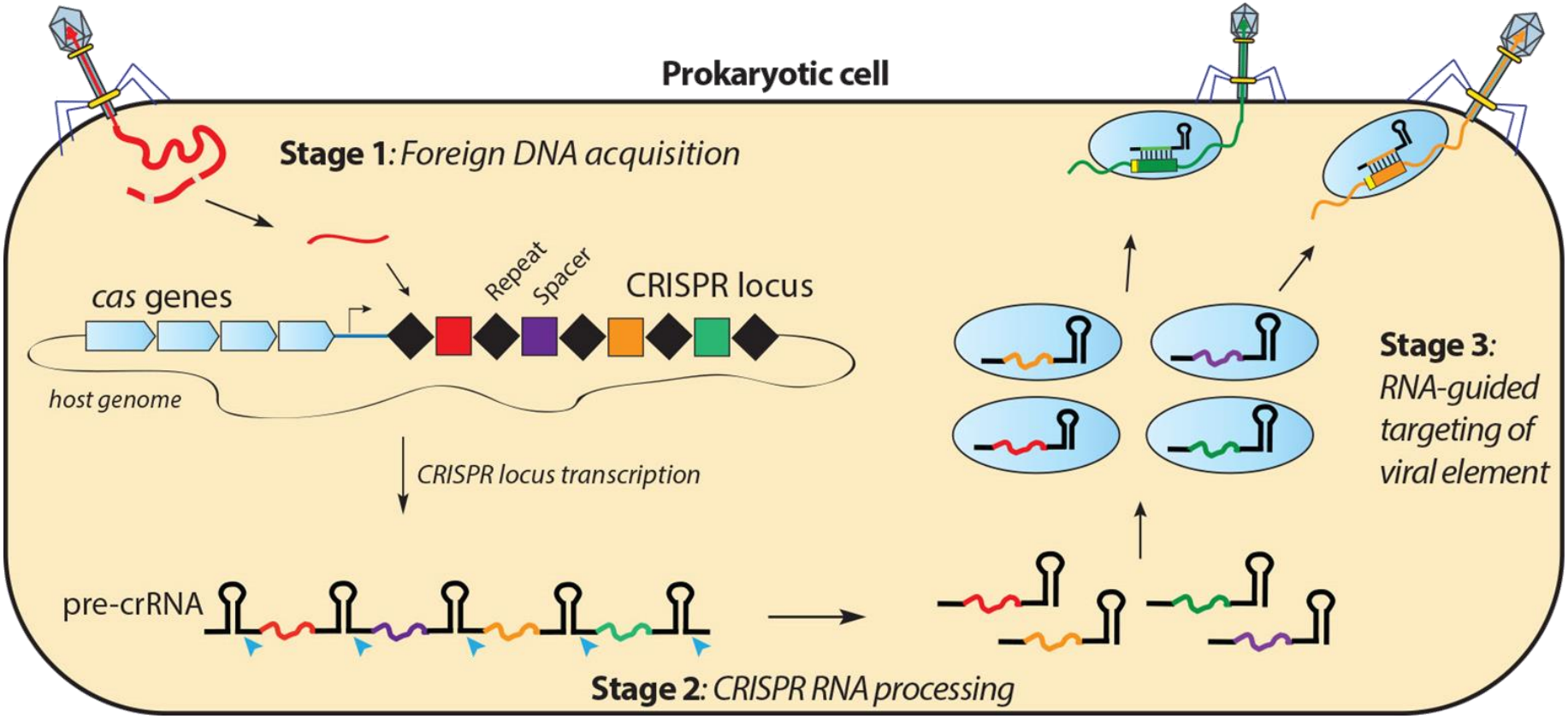
Innate

- 1970's
- Restriction Enzymes + DNA methyltransferases
- 4-8bp non-programmable recognition site.

Adaptive

- 2012-now
- CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)

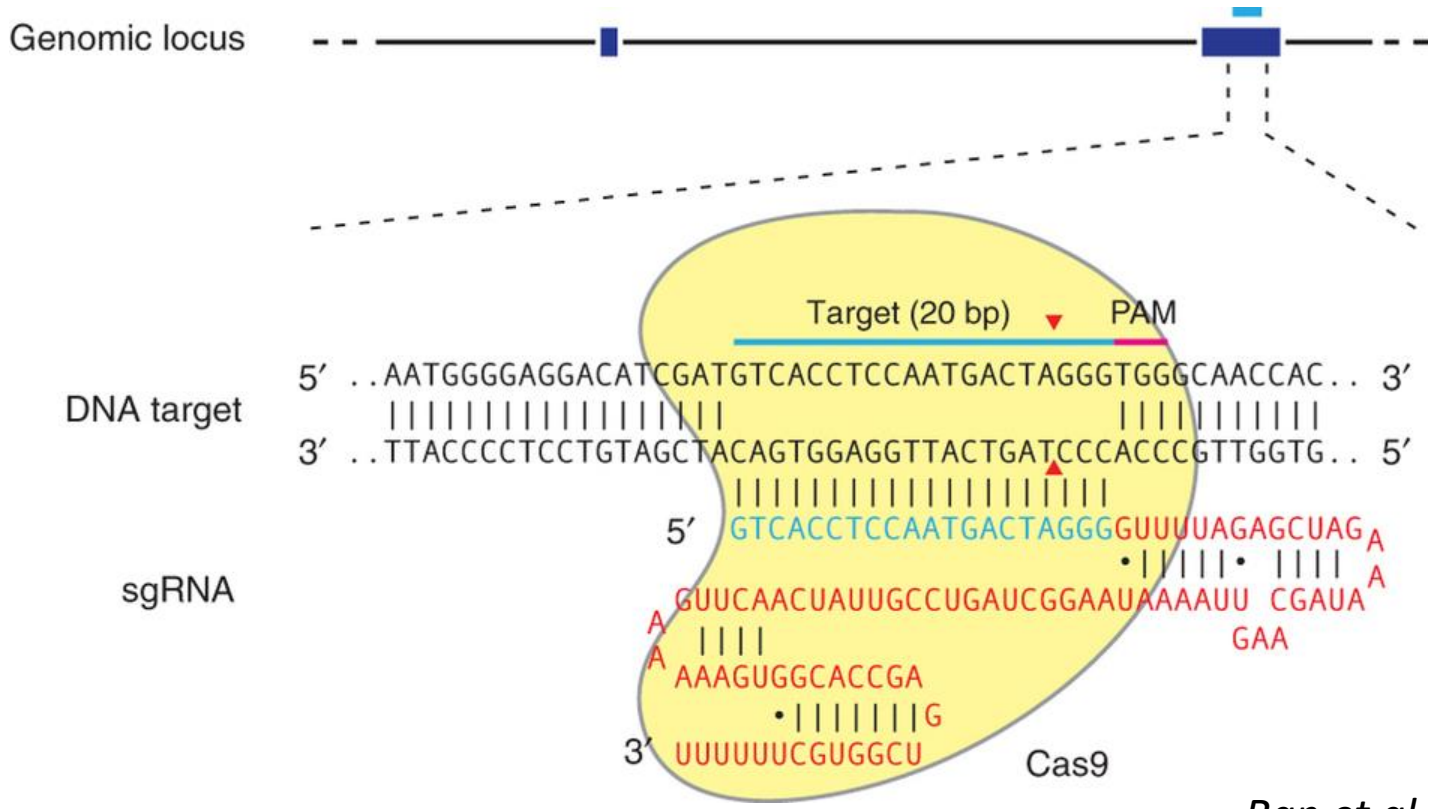
Role of CRISPR in Bacterial Immunity



Doudna Lab

Cas9-mediated Genome Editing

- RNA-directed protein DNAse (Cas9 + sgRNA)
- Species independent



Ran et al, Nat Prot. 2013

Repurposing Bacterial Immune Systems

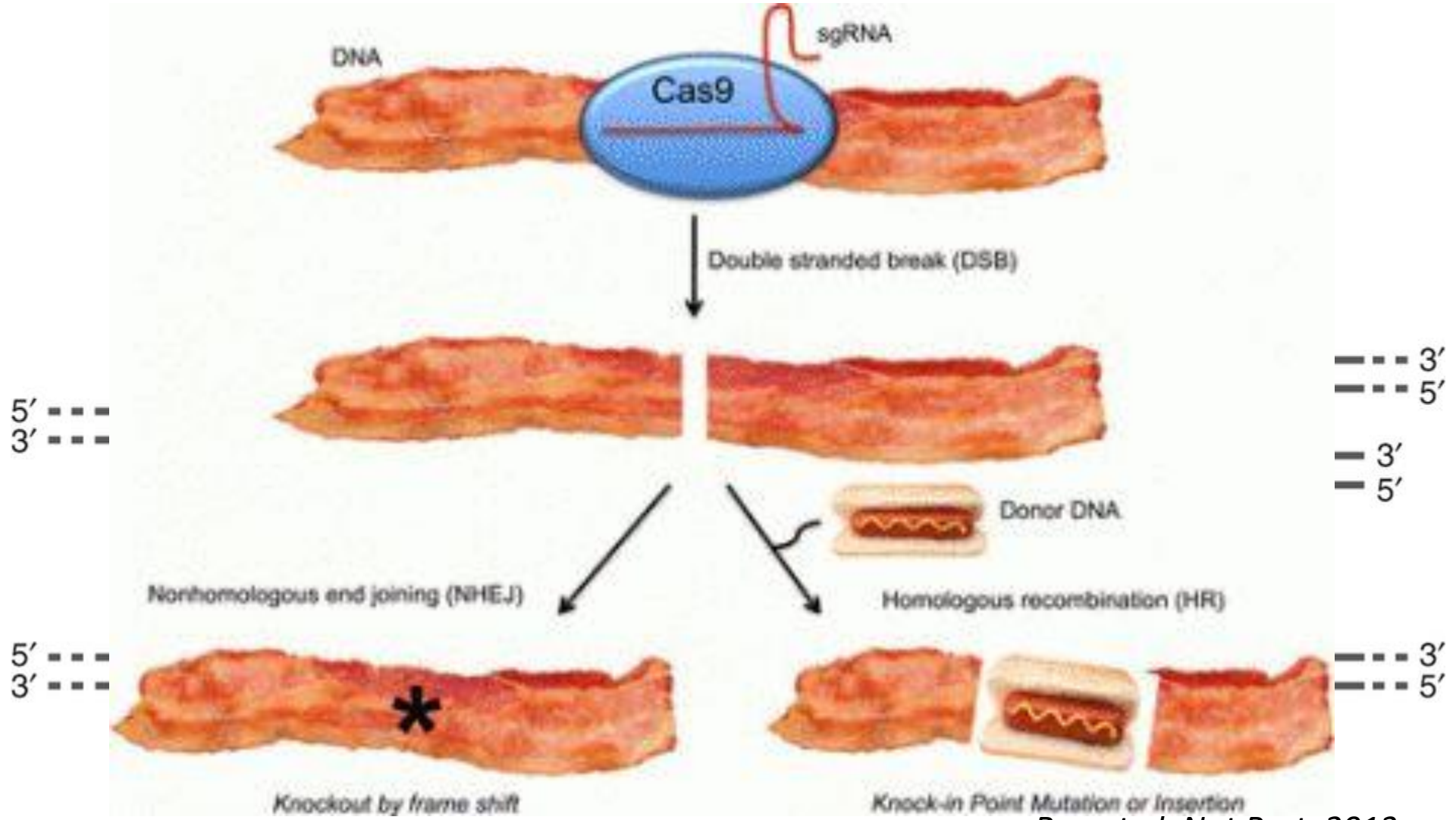
Innate

- 1970's
- Restriction Enzymes + DNA methyltransferases
- 4-8bp non-programmable recognition site. (1/32kb = ~100,000 sites in the haploid genome)

Adaptive

- 2012-now
- CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)
- ~20bp programmable recognition site (17bp necessary for uniqueness on average)

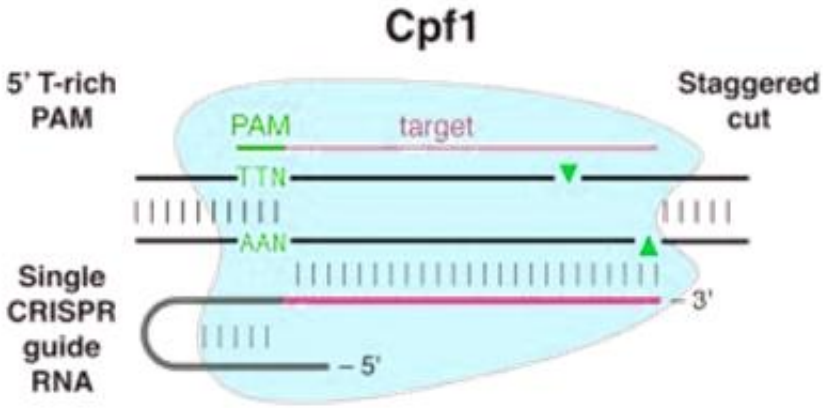
Cas9-mediated Genome Editing



Ran et al, Nat Prot. 2013

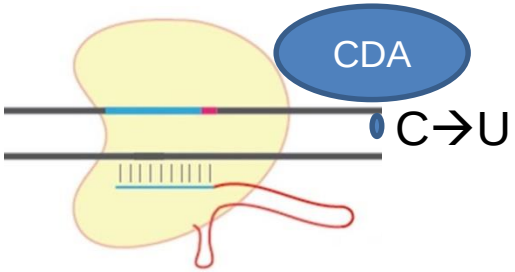
Cas9-mediated Genome Editing Broadening the Target Range

Species/Variant of Cas9	PAM Sequence
Strep. pyogenes; SpCas9	N(G)G
SpCas9 D1135E	NGG
SpCas9 VRER variant	NGCG
SpCas9 EQR variant	NGAG
SpCas9 VQR variant	NGAN or NGNG
Staph. aureus (Sa)	NNGRR(T)
Neisseria meningitidis (Nm)	NNNNGATT
Strep. thermophilus (St)	NNAGAAW
Treponema denticola (Td)	NAAAAC

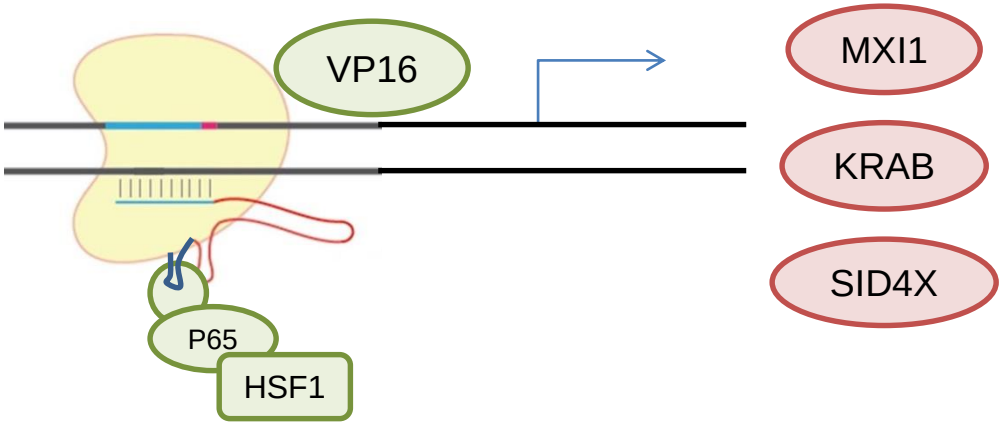


Cas9-mediated Genome Editing
Broadening the Functional Range with dCas9

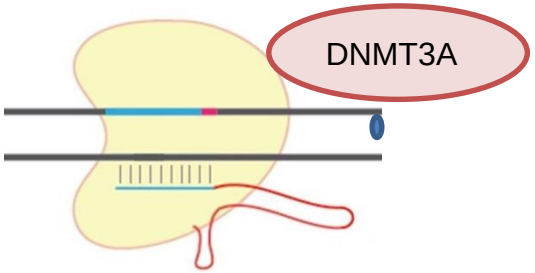
Single-base editing



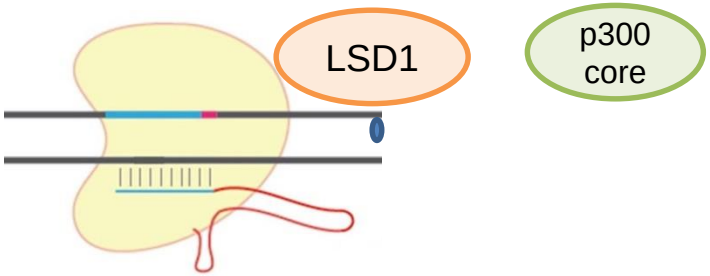
Transcriptional Regulation



DNA Methylation



Histone Modification

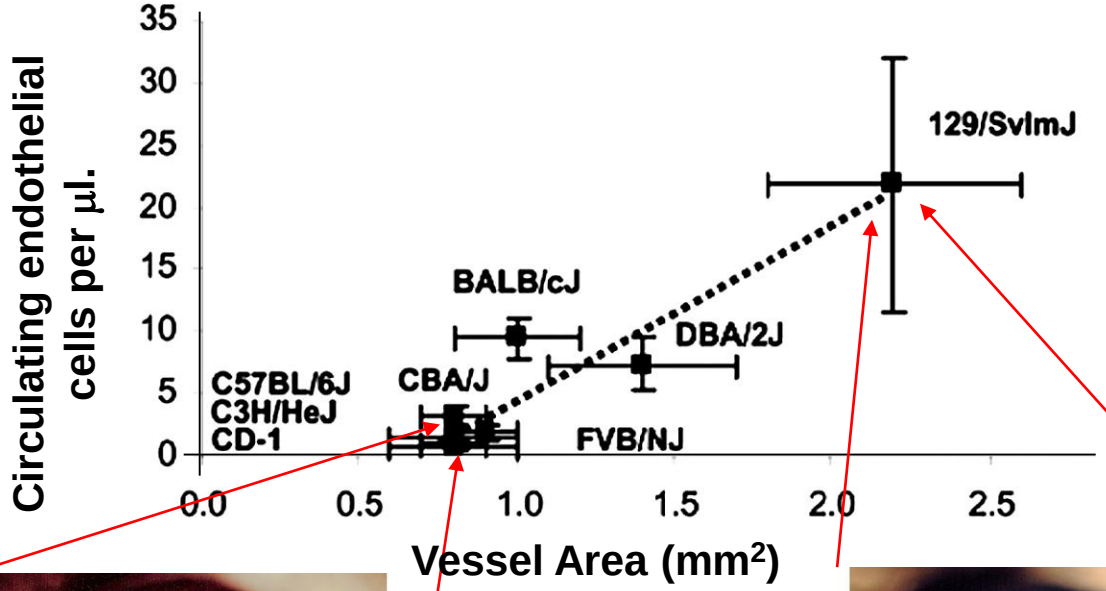


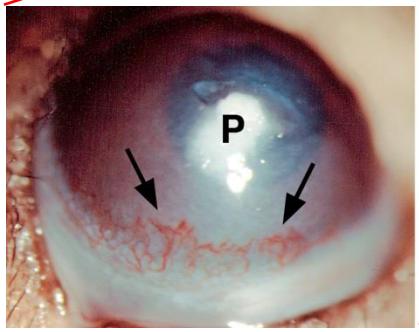
. . .

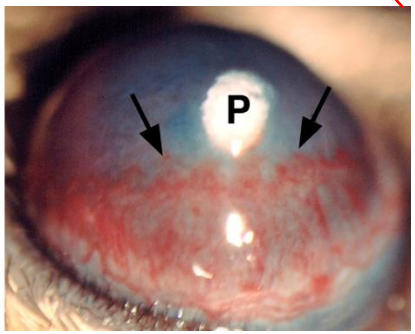
How Did I Get Here?

Mice (and Humans?) Vary Dramatically in their Angiogenic Response

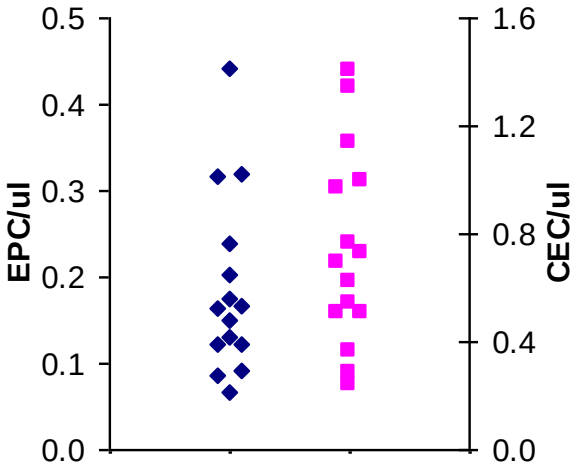
Mouse







Human



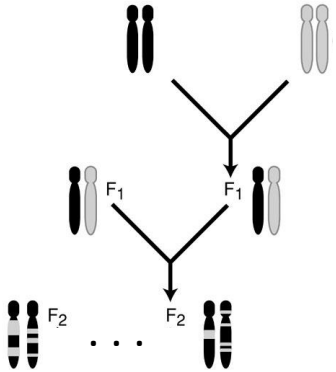
Anat Norden-Zfoni &
John Heymach, unpublished

Cancer Cell, 7: 101, 2005.

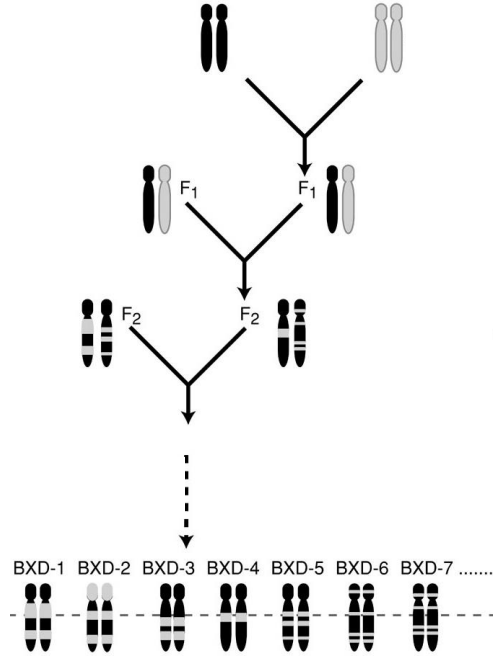
Which Polymorphism(s) Affect Host Neovascular Response?

Strategies Used to Identify Angiogenesis QTLs

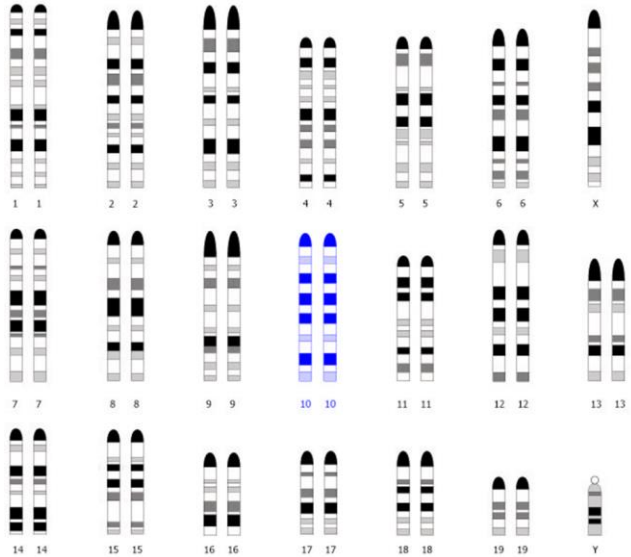
F2 Intercross



Recombinant Inbred Cross



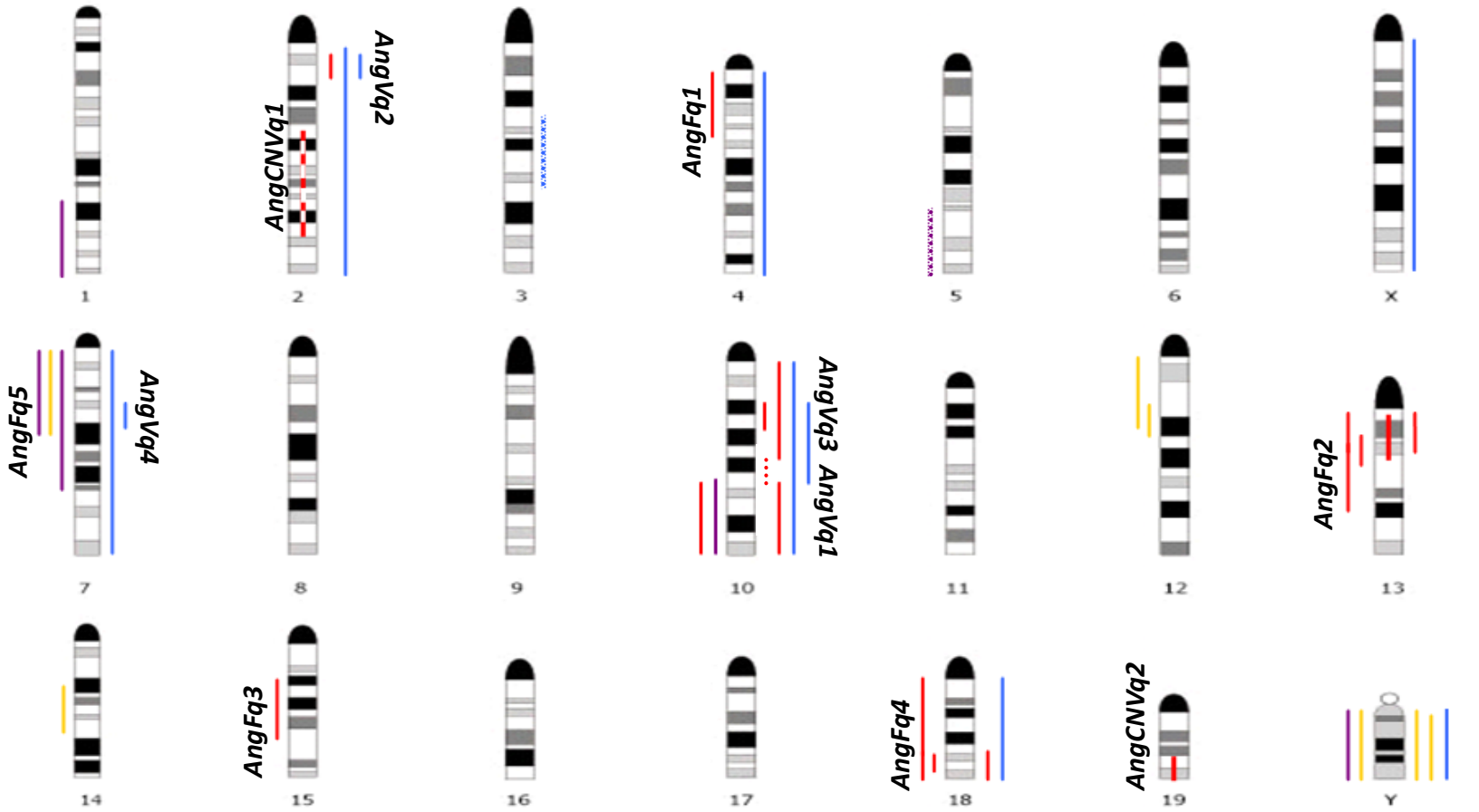
Consomic Inbred Strains



Composite Interval Mapping

$$y_i = \mu + b^* x_i^* + \sum_k b_k x_{ik} + e_i$$

Angiogenic-responsiveness-linked Regions



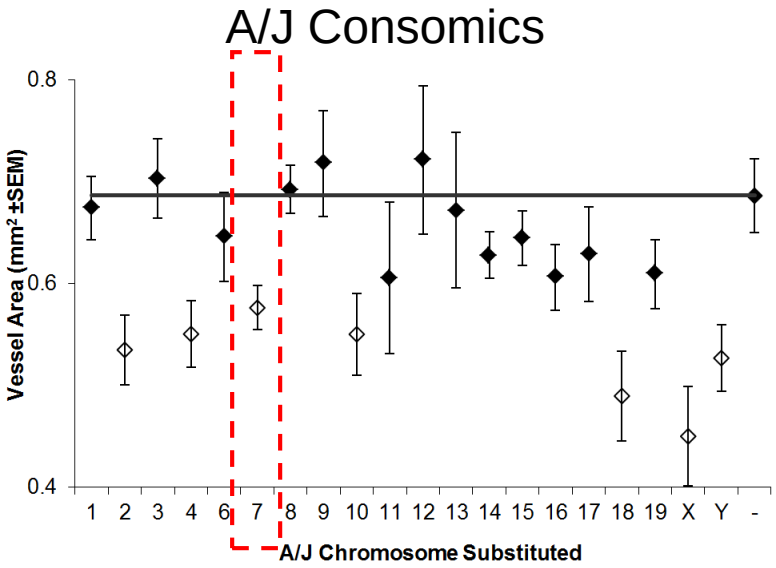
Left bFGF-linked
Right VEGF-linked
Center CNV-linked

Decrease
Increase

DBA/2J
A/J

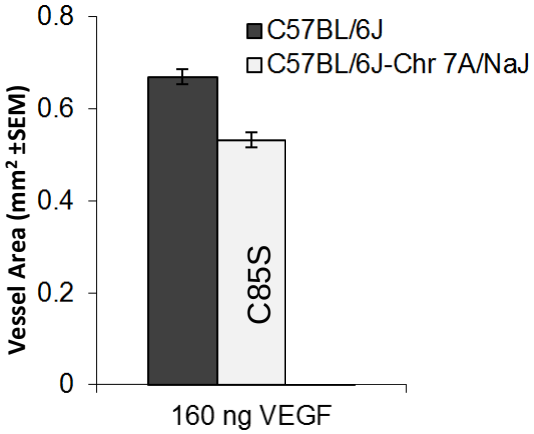
129
SJL/J

Identification of *AngVq4*

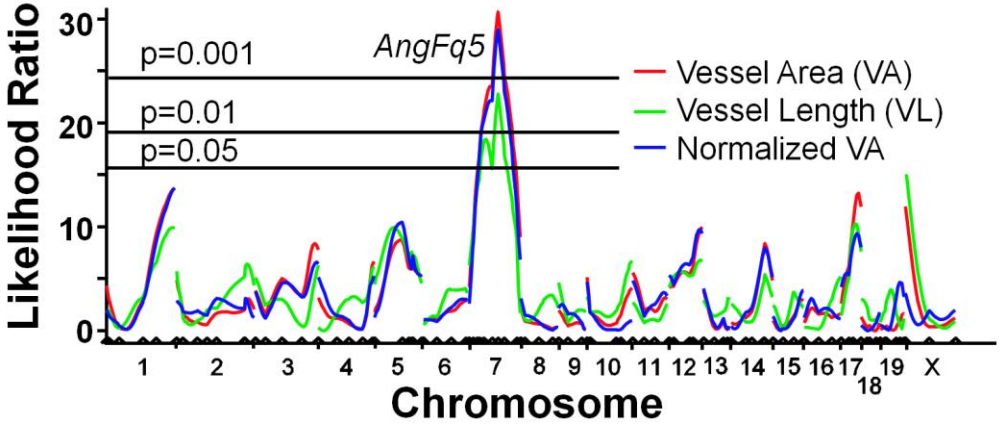


Identification of *AngVq4*

A/J Consomics

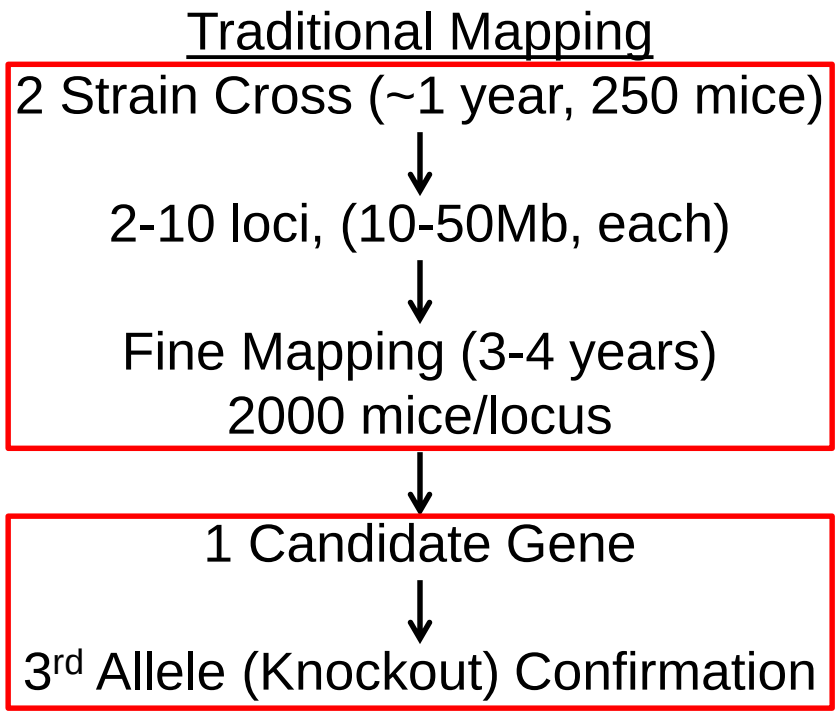


Oca2^{p} Can Explain *AngFq5*



The *pJ* allele arose independently of the classical *p* allele.

Can We Speed this Up?

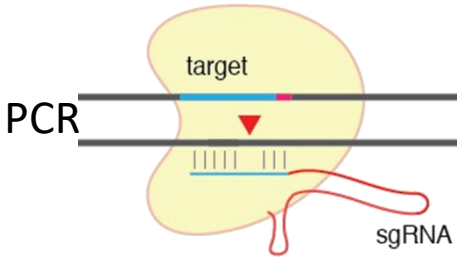


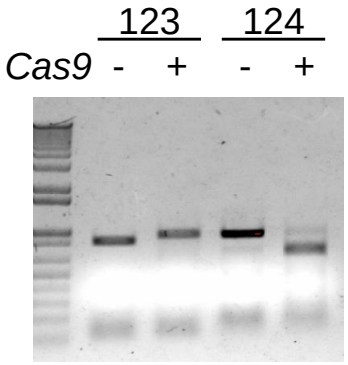
- GWAS
- Smaller Regions
- Wider Genomic Variety Samples
- Still Requires Confirmation

- Threshold for Knockout Generation has Historically been High (~$50k)
- Inefficient Use of Negative Results

Strategy Used

1. Select pigment-production genes
2. Clone tru-sgRNA into pX459, PCR, and *in vitro* transcribe
3. Assess activity of sgRNA on PCR fragments





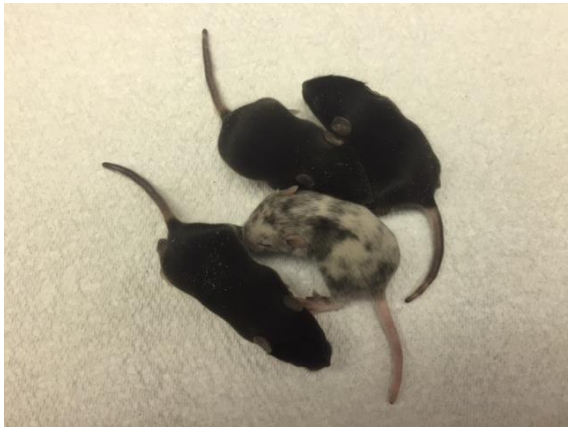
7/11 Primary sgRNA active
5/5 Secondary sgRNA active

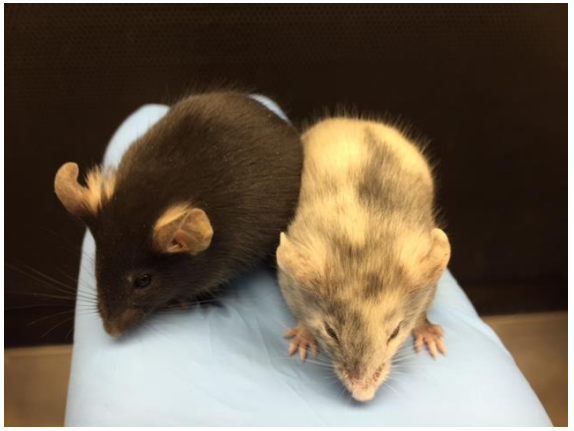
Gene	Ch	Phenotype	sgRNA
Mreg	1	Dilute suppressor	
Atrn	2	Mahogany	
Tyrp1	4	Brown	
Vps33a	5	Buff	
Oca2	7	Pink-eyed dilute	
Mc1r	8	Extension (red)	
Drd2	9	Dark agouti	
Pmel	10	Silver	
Pomc	12	Red	
Bloc1s5	13	Muted	
Dct	14	Slaty	
Slc45a2	15	Underwhite	

Table I. Targeted mouse genes. Ch, chromosome; sgRNA, results of *in vitro* cleavage - *Cas9*, + *Cas9*.

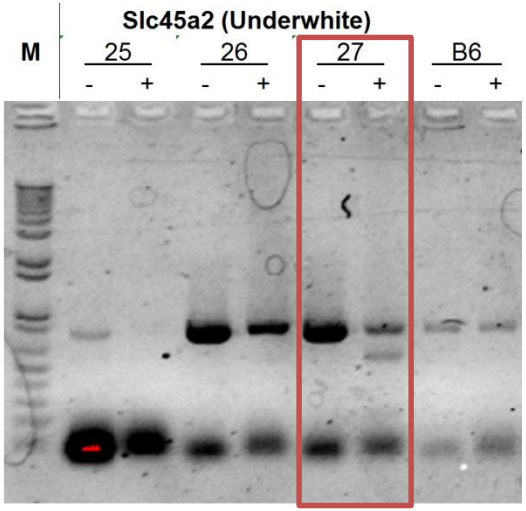
4. Mouse zygote injection
5. Screen by coat color, T7EI digest, sequence.

CRISPR-Generated Underwhite Alleles (*Slc45a2*)



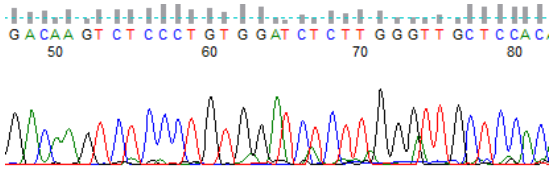


T7 Endonuclease 1 Digest

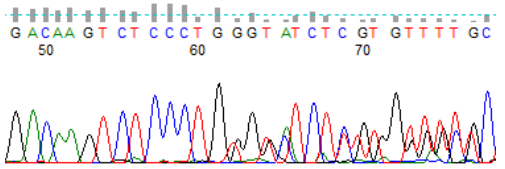


Sanger Sequencing of F1 Progeny

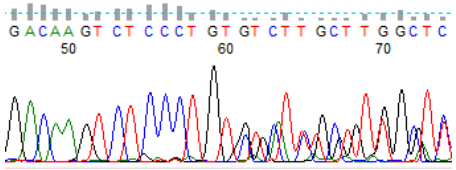
C57BL/6J



27-1 (+/-2)



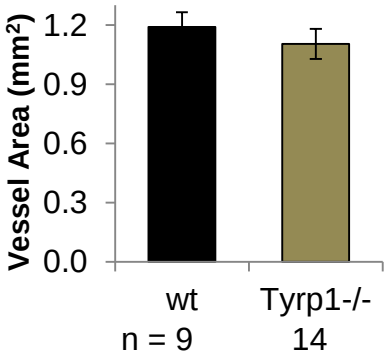
27-3 (+/-4)

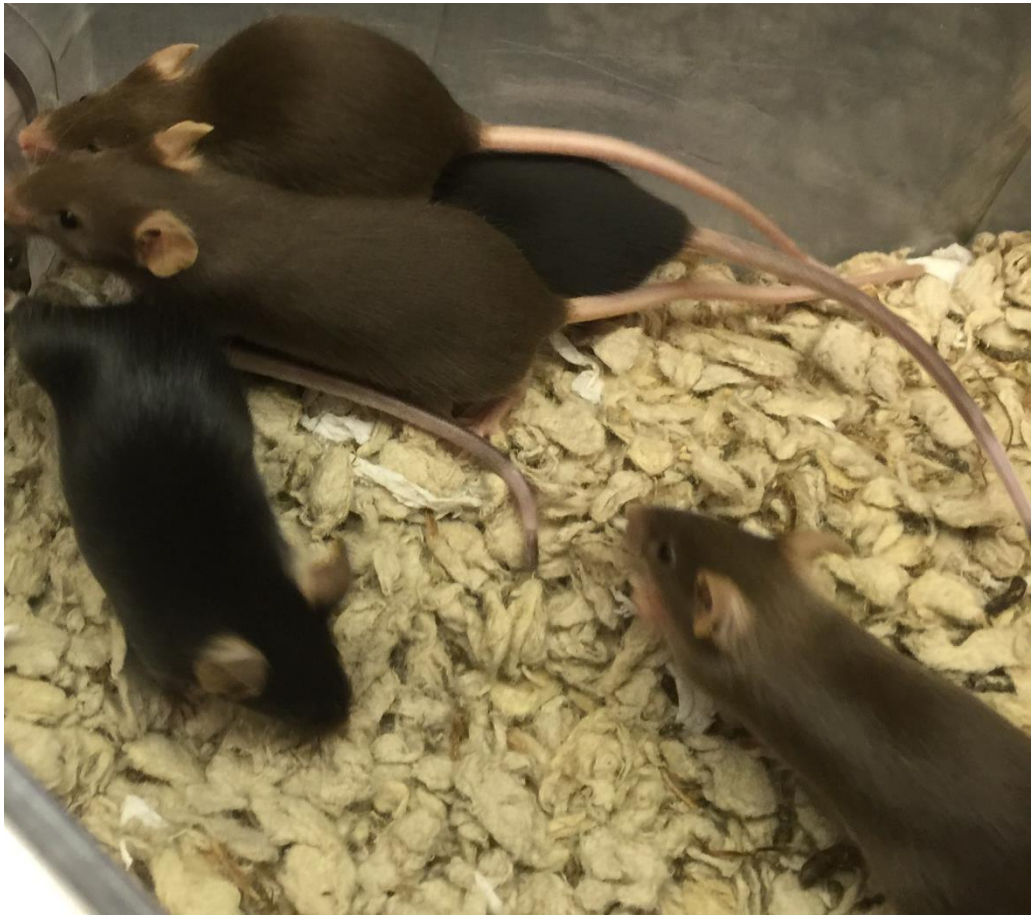


. . . and Brown Alleles (*Tyrp1*)

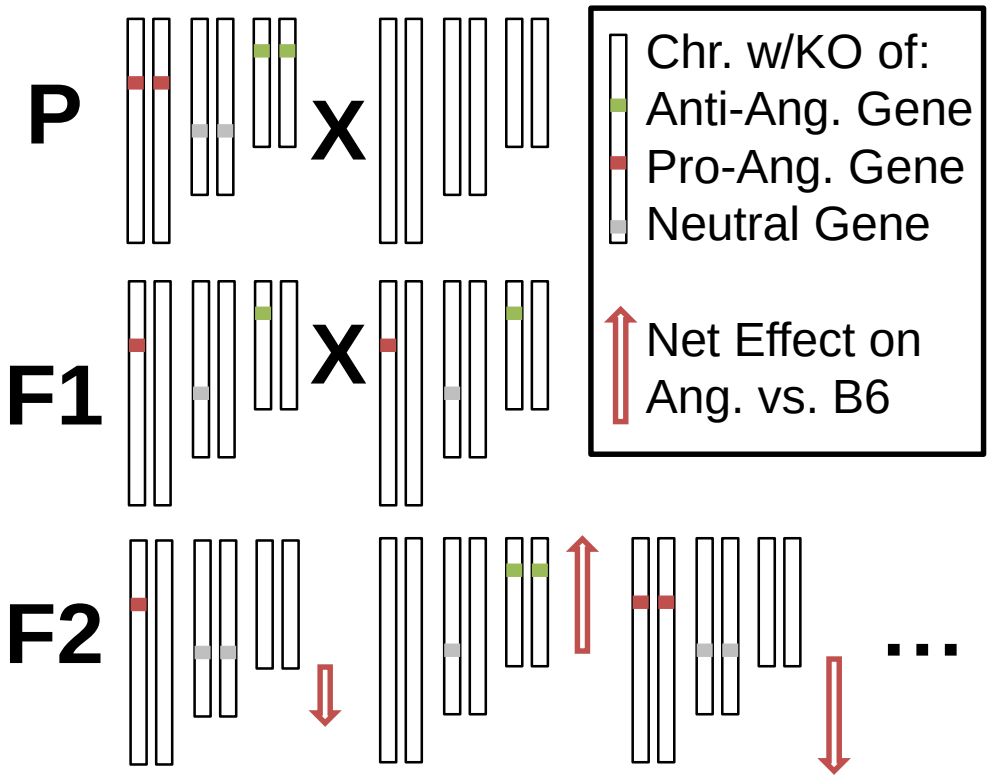
Mouse	sgRNA (cut site) 121	122
121/122-8	wt/wt	+1/+1
121/122-9	wt/wt	-10/-14/c/
121/122-10	+480/+480	???
121/122-11	wt/wt	+1/-7
121/122-12	-1/-5	-3/-6
121/122-13	wt/wt	wt/wt
121/122-14	wt/wt	wt/wt
121/122-15	wt/wt	wt/wt

Inserted sequence corresponds to a fragment of a murine endogenous retrovirus.





Multiplex Genome Targeting (mGeT)



Advantages of mGeT

- Fewer Mice→Cheaper

	1 Gene	x4	mGeT (12)
Parameters/gene	2		24
Epistasis	0		12
Total Parameters	2		36
P	7+2	28+8	10
F1	10	40	30
F2 (α=0.05, β=0.2)	31	124	104
Total Animals	50	200	144

Power analysis using G*Power, Large effect size (f2=0.35),
Effect, dominance for each gene, Epistasis tested only on positives

- Built-in controls (not all candidates will be active)
- Built-in identification of epistasis

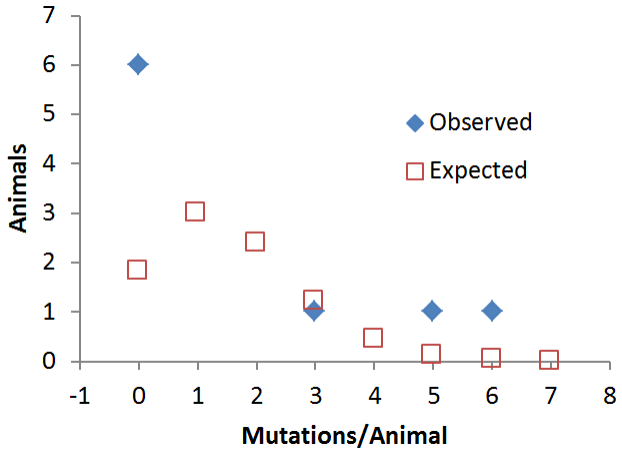
Genetic architecture of complex traits: Large phenotypic effects and pervasive epistasis

Haifeng Shao[a,b,1], Lindsay C. Burrage[a,b,1], David S. Sinasac[a,1], Annie E. Hill[a], Sheila R. Ernest[a], William O'Brien[c], Hayden-William Courtland[d], Karl J. Jepsen[d], Andrew Kirby[e], E. J. Kulbokas[e], Mark J. Daly[e,f], Karl W. Broman[g], Eric S. Lander[f,h,i,2,3], and Joseph H. Nadeau[a,b,j,k,2,3]

PNAS 105:19910 (2008)

Multiplex Genome Targeting (mGeT)

	gene	len	phenotype	% hit	24	25	26	27	12.13-1	12.13-2	12.13-3	12.13-4	12.13-5
101	Atrn-1	19	mahogany	0%	wt/wt	wt/wt	wt/wt	wt/wt					
102	Atrn-2	20	mahogany	0%	wt/wt	wt/wt	wt/wt	wt/wt					
103	Bloc1s5-1	20	*Muted*	0%	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt
105	Dct-1	20	slaty	0%	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt
108	Drd2-2	19	dark agouti	0%	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt
109	Mc1r-1	20	extension	5%	wt/wt	wt/wt	wt/wt	wt/+1/c	wt/-3	wt/wt	wt/wt	wt/wt	wt/wt
112	Mreg-2	20	dilute suppressor	16%	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt
114	Oca2-2	20	pink-eyed dilute	15%	wt/wt	wt/wt	wt/wt	wt/-16	wt/-1	wt/wt	wt/wt	wt/wt	wt/wt
115	Pmel-1	20	silver	20%	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt
117	Pomc-1	20	Red	11%	wt/wt	wt/wt	wt/wt	++c	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt
119	Slc45a2-1	19	underwhite	33%	wt/wt	+1/+1-13	wt/wt	++c	-9/-9	wt/wt	wt/wt	wt/wt	wt/wt
121	Tyrp1-1	19	brown	12%	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt
122	Tyrp1-2	19	brown	24%	wt/wt	wt/wt	wt/wt	wt/wt	wt/-11	wt/wt	wt/wt	wt/wt	wt/wt
124	Vps33a-2	20	buff	3%		wt/-14	*wt/wt*	*wt/wt*	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt



CRISPR-induced mutations are <u>not</u> randomly distributed (P<0.001). If one allele is mutated, other are more likely to be.

Does early targeting tend to result in homozygotes? (P=0.11)

Multiplex Genome Targeting (mGeT)

	gene	len	phenotype	% hit	24	25	26	27	12.13-1	12.13-2	12.13-3	12.13-4	12.13-5
101	Atrn-1	19	mahogany	0%	wt/wt	wt/wt	wt/wt	wt/wt					
102	Atrn-2	20	mahogany	0%	wt/wt	wt/wt	wt/wt	wt/wt					
103	Bloc1s5-1	20	*Muted*	0%	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt
105	Dct-1	20	slaty	0%	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt
108	Drd2-2	19	dark agouti	0%	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt
109	Mc1r-1	20	extension	5%	wt/wt	wt/wt	wt/wt	wt/+1/c	wt/-3	wt/wt	wt/wt	wt/wt	wt/wt
112	Mreg-2	20	dilute suppressor	16%	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt
114	Oca2-2	20	pink-eyed dilute	15%	wt/wt	wt/wt	wt/wt	wt/-16	wt/-1	wt/wt	wt/wt	wt/wt	wt/wt
115	Pmel-1	20	silver	20%	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt
117	Pomc-1	20	Red	11%	wt/wt	wt/wt	wt/wt	++c	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt
119	Slc45a2-1	19	underwhite	33%	wt/wt	+1/+1-13	wt/wt	++c	-9/-9	wt/wt	wt/wt	wt/wt	wt/wt
121	Tyrp1-1	19	brown	12%	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt
122	Tyrp1-2	19	brown	24%	wt/wt	wt/wt	wt/wt	wt/wt	wt/-11	wt/wt	wt/wt	wt/wt	wt/wt
124	Vps33a-2	20	buff	3%		wt/-14	*wt/wt*	*wt/wt*	wt/wt	wt/wt	wt/wt	wt/wt	wt/wt

Are targeted alleles efficiently passed on to progeny?

Late targeting results in germline chimerism (>2 alleles/mouse).

Can We Speed this Up?

<u>Traditional Mapping</u>

2 Strain Cross (~1 year, 250 mice)

↓

2-10 loci, (10-50Mb, each)

↓

Fine Mapping (3-4 years)
2000 mice/locus

↓

1 Candidate Gene

↓

3rd Allele (Knockout) Confirmation

<u>5-6 years, 2500 mice, 1 Gene</u>

<u>Faster Alternative</u>

GWAS (~1 year, ~250 mice)

↓

2-10 loci, (20-100kb, each)

↓

mGeT (1 year)
200-400 mice

↓

2-4 Genes

↓

4th Allele/Knockin Confirmation

<u>3 years, 1000 mice, 2-4 Genes</u>

Conclusions

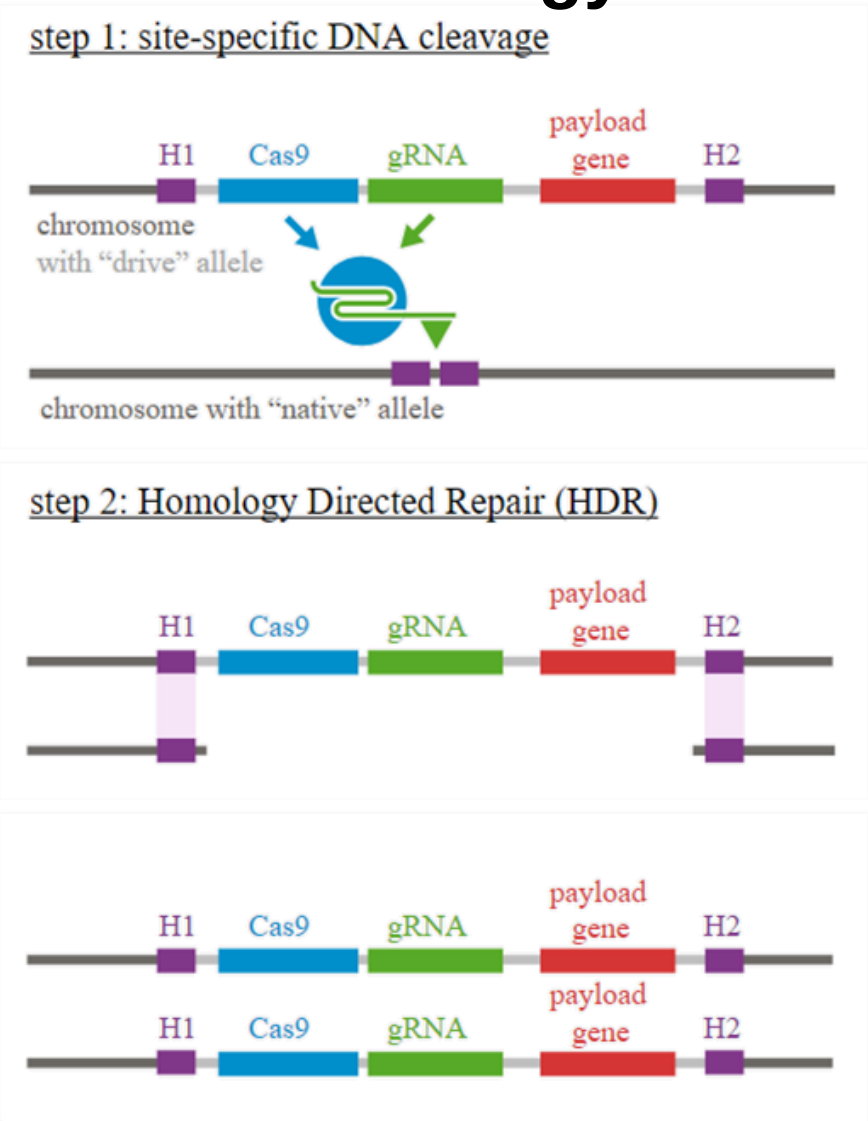
- Host angiogenic response is a multigenic trait.
- Variants in pigment production genes affect the host angiogenic response.
- CRISPR-based multiplex genome editing has promise to reduce the cost of confirming mapped genes.
- CRISPR-induced mutation efficiency is non-randomly distributed.

Now What?

Uses for CRISPR Genome Editing

Applications of CRISPR Technology

- Food Production
  - Livestock modification
  - Crop improvement
  - Improved microbes
- Industrial Use
  - Feedstock production (esp. Pharm)
  - Fossil fuel alternatives
- Pest control (gene drive)



Potential Human Therapeutic Applications

- Livestock modification to improve suitability for transplantation

- *Ex vivo* genome editing
  - NHEJ—CCR5 knockout for HIV
  - HDR—ADA for SCID, etc.

- *In vivo* genome editing
  - NHEJ—oncogene knockout for cancer
  - HDR—repair of dystrophin for DMD

- Germline genome editing
  - NHEJ—reduce pathogenicity of nt repeats (Huntington's disease).
  - HDR—repair of Mendelian recessive disease alleles (cystic fibrosis, etc.)

BIOTECHNOLOGY

A prudent path forward for genomic engineering and germline gene modification

A framework for open discourse on the use of CRISPR-Cas9 technology to manipulate the human genome is urgently needed

By **David Baltimore,[1] Paul Berg,[2] Michael Botchan,[3,4] Dana Carroll,[5] R. Alta Charo,[6] George Church,[7] Jacob E. Corn,[4] George Q. Daley,[8,9] Jennifer A. Doudna,[4,10]* Marsha Fenner,[4] Henry T. Greely,[11] Martin Jinek,[12] G. Steven Martin,[13] Edward Penhoet,[14] Jennifer Puck,[15] Samuel H. Sternberg,[16] Jonathan S. Weissman,[4,17] Keith R. Yamamoto[4,18]**

ture developments. The meeting identified immediate steps to take toward ensuring that the application of genome engineering technology is performed safely and ethically.

The promise of so-called "precision medicine" is propelled in part by synergies between two powerful technologies: DNA sequencing and genome engineering. Advances in DNA sequencing capabilities and genome-wide association studies have

CURRENT APPLICATIONS. The simplicity of the CRISPR-Cas9 system allows any researcher with knowledge of molecular biology to modify genomes, making feasible experiments that were previously difficult or impossible to conduct. For example, the CRISPR-Cas9 system enables introduction of DNA sequence changes that correct genetic defects in whole animals, such as replacing a mutated gene underlying

1) Strongly discourage, . . . attempts at (human) germline genome modification . . . while societal, environmental, and ethical implications of such activity are discussed among scientific and governmental organizations. This will enable pathways to responsible uses of this technology, if any, to be identified.

2) Create forums (for education on risks/rewards).

3) Encourage and support transparent research . . . (efficacy and specificity).

4) (Meet again) . . . and where appropriate, recommend policies.

International Summit on Human Gene Editing (1-3 December 2015)

"It would be irresponsible to proceed with any clinical use of germline editing unless and until

(i) the relevant safety and efficacy issues have been resolved, based on appropriate understanding and balancing of risks, potential benefits, and alternatives, and

(ii) there is broad societal consensus about the appropriateness of the proposed application.

Moreover, any clinical use should proceed only under appropriate regulatory oversight. At present, these criteria have not been met for any proposed clinical use: the safety issues have not yet been adequately explored; the cases of most compelling benefit are limited; and many nations have legislative or regulatory bans on germline modification. However, as scientific knowledge advances and societal views evolve, the clinical use of germline editing should be revisited on a regular basis."

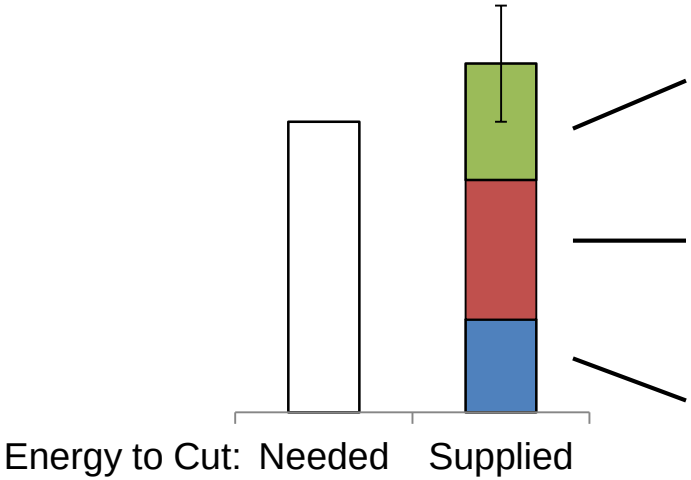
Challenges to Safe and Effective Genome Editing

Technical

- Fidelity
  - On target activity

Increasing Fidelity (Decreasing off-target)

Energy Management

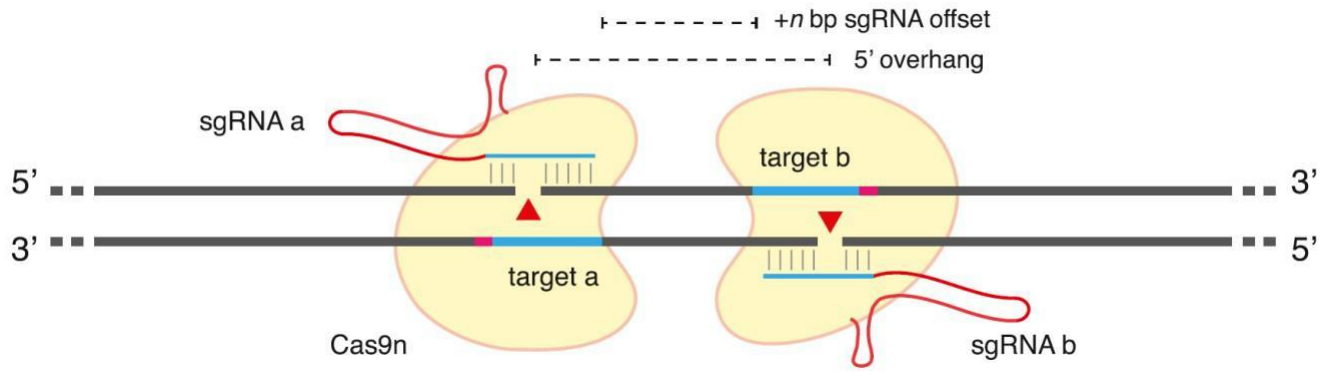


gRNA-Target-strand
tru-sgRNAs (18-mers)

Protein-Non-target-strand
eSpCas9(1.1) (K848A,K1003A,R1060A)
eSaCas9 (R499A,Q500A,R654A,G655A)

Protein-Target-strand
SpCas9-HF1 (N497A,R661A,Q695A,Q926A)

Dual Nickase



Challenges to Safe and Effective Genome Editing

Technical

- Fidelity
  - On target activity
  - Expected repair templates (vs. ERVs)
- Efficiency (homology-directed repair)
- Timing (chimera production)

Conceptual

- Knowledge (How certain are we about the effects of specific mutations in a new haplotype context?)
- Pleiotrophy (e.g. coat color genes and angiogenesis)

Acknowledgements

- **Robert D'Amato**
- Lauren Bazinet
- Amy Birsner
- Dema Luyindula
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- Huali Yin
- Jenny Yuan
- Jessica Stiles
- Mantu Bhaumik

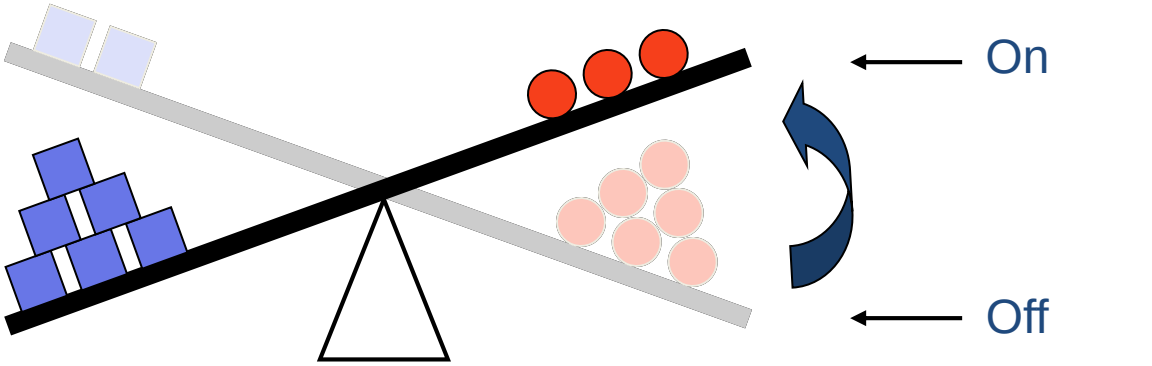
- **Bill Dietrich**
- Victor Boyartchuk

- Judah Folkman
- Harold Dvorak

- Funding from NEI
- BCH VBP Funding

The Balance Hypothesis for the Angiogenic Switch

The switch:



Activators

bFGF
aFGF
VEGF
-
-
-
-
-
-

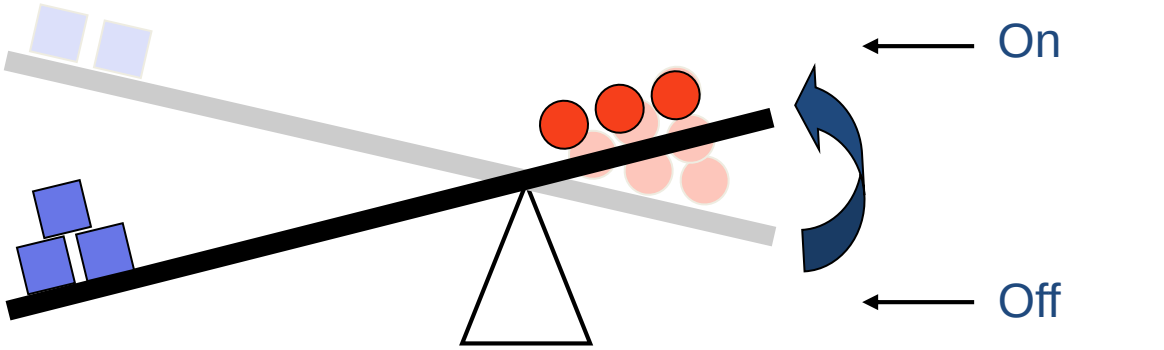
Inhibitors

Thrombospondin-1
16 kD Prolactin
Interferon a/b
Platelet factor-4
Angiostatin
-
-
-

Folkman and Hanahan, Cell 1996.

The Balance Hypothesis for the Angiogenic Switch

The switch:



Activators

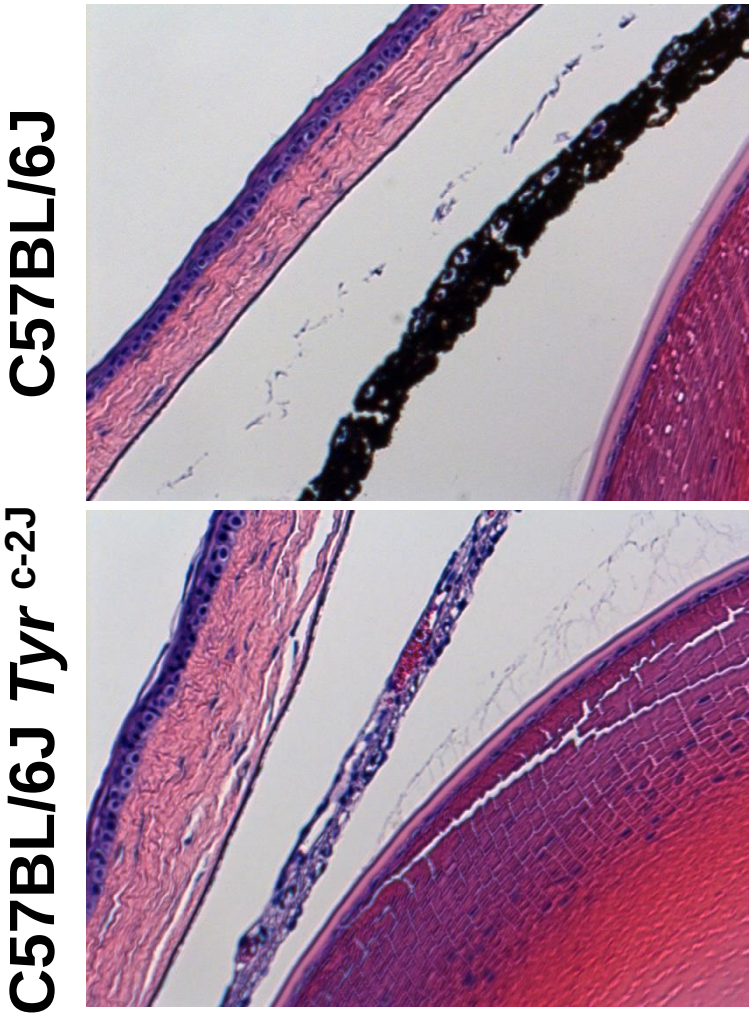
bFGF
aFGF
VEGF
-
-
-
-
-
-

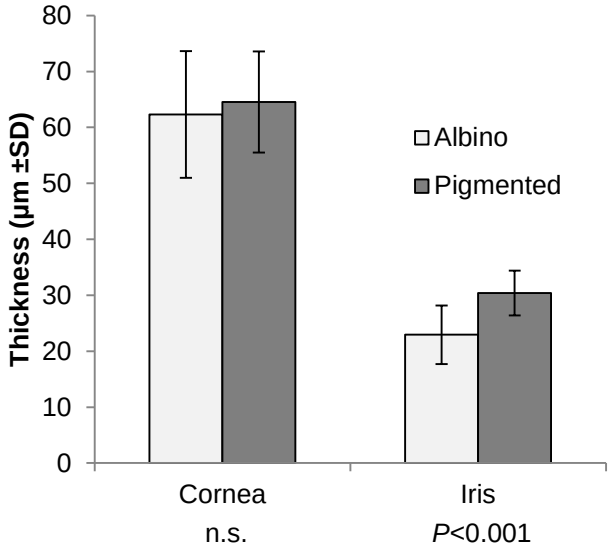
Inhibitors

Thrombospondin-1
16 kD Prolactin
Interferon a/b
Platelet factor-4
Angiostatin
-
-
-

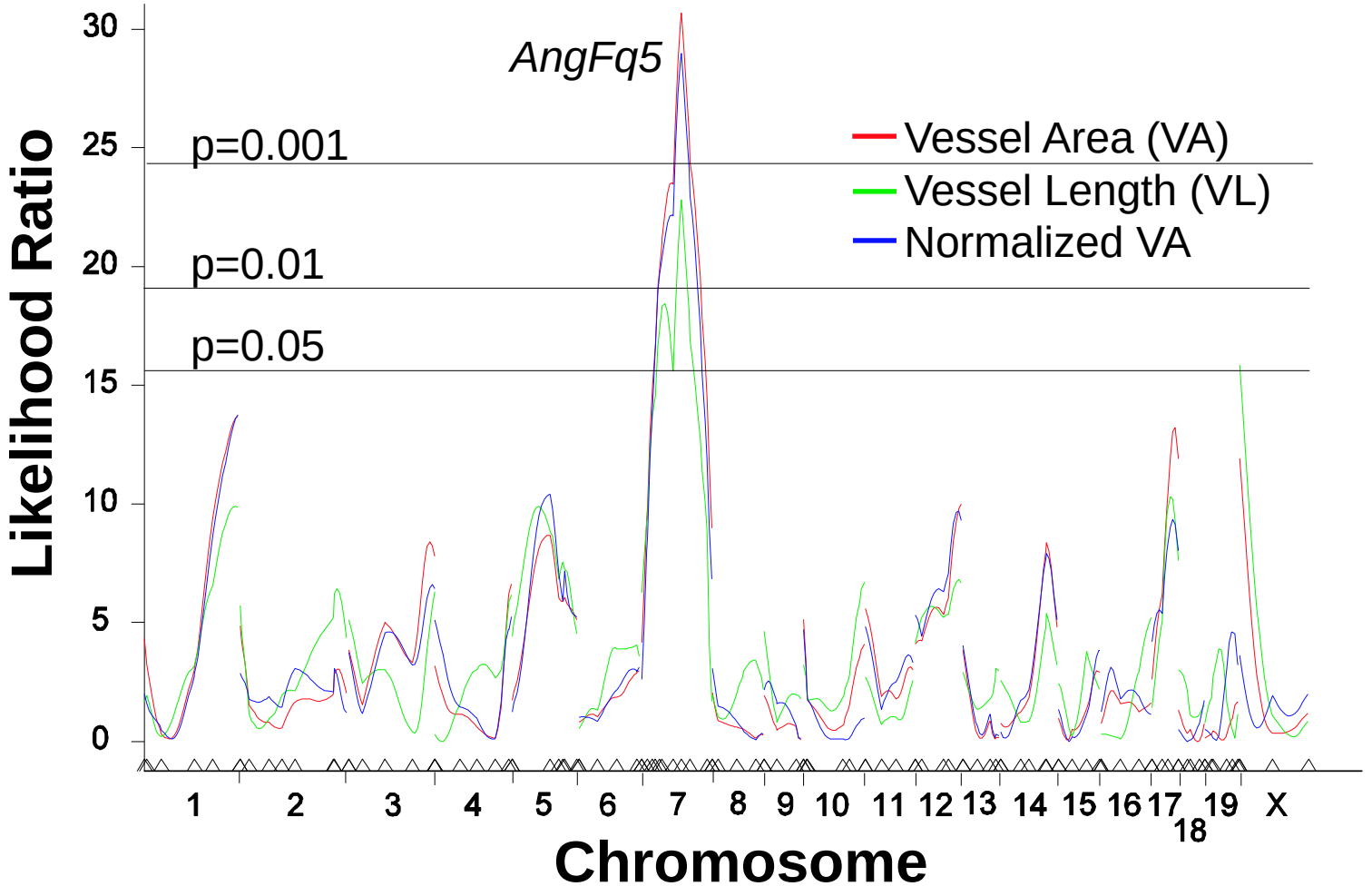
Folkman and Hanahan, Cell 1996.

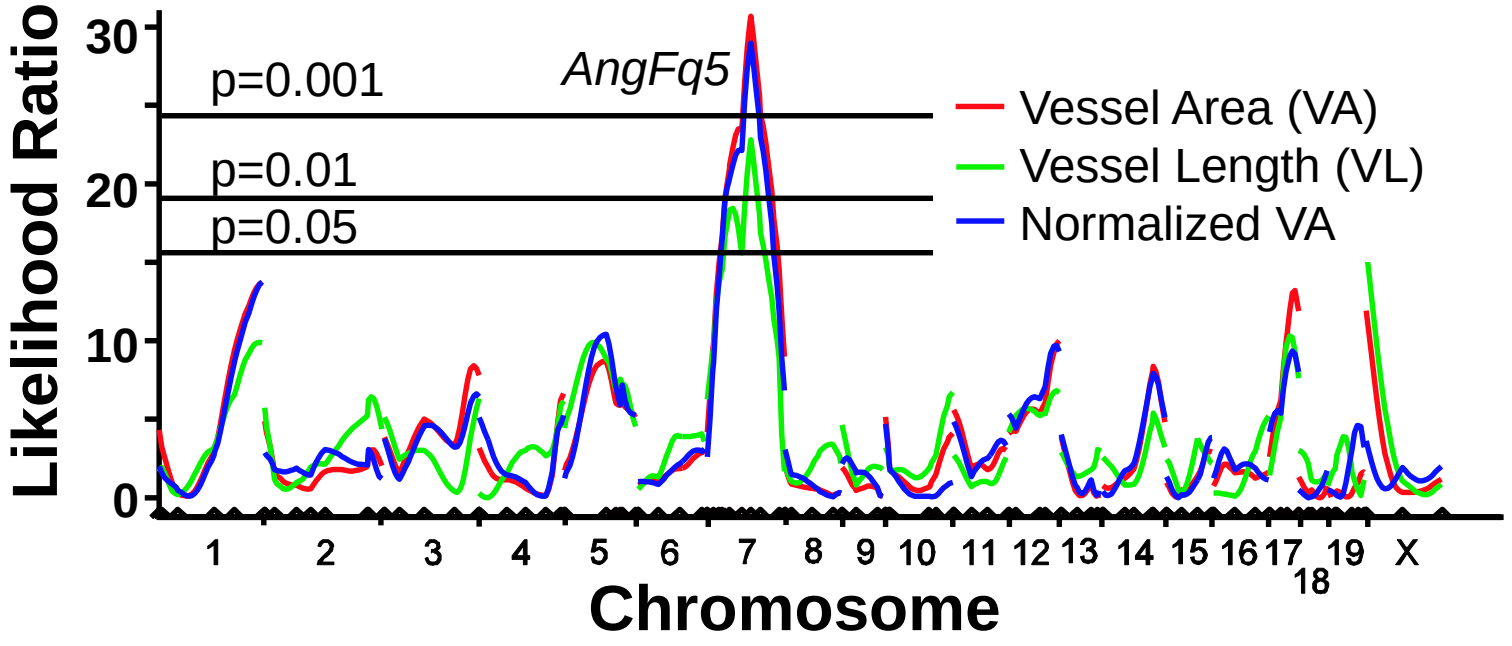
Structural Differences Cannot Explain Differences in Corneal Neovascularization





Eyes from 10 week old C57BL/6J and C57BL/6J<tyr-C2J> mice, fix, section and stain (H&E and Masson's trichrome). Capture images and measure corneal thickness twice on 2 sections ~ ½ way between limbus and centerline. Similarly measure iris thickness ~½way between pupil and margin.


AngFq5
p=0.001
p=0.01
p=0.05
Vessel Area (VA)
Vessel Length (VL)
Normalized VA
Likelihood Ratio
30
25
20
15
10
5
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
X
Chromosome


Likelihood Ratio
30
20
10
0
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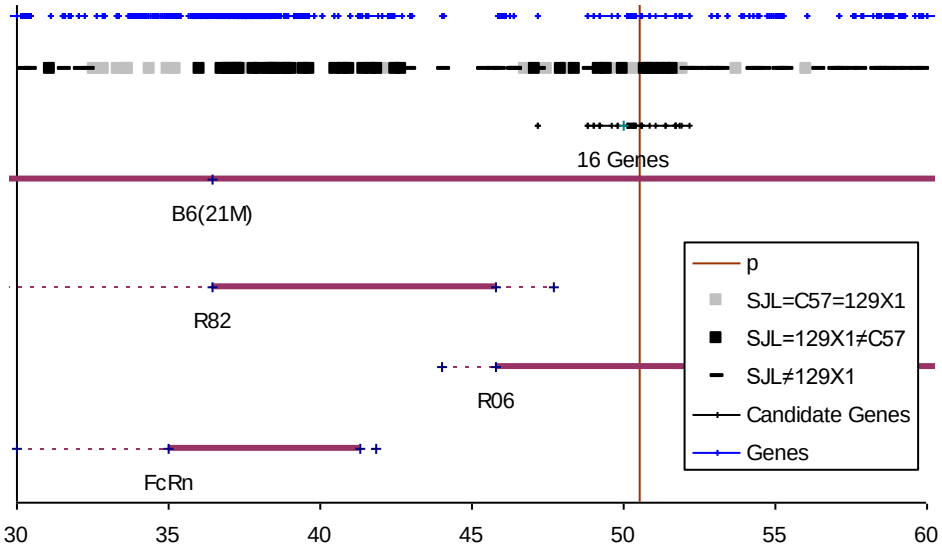
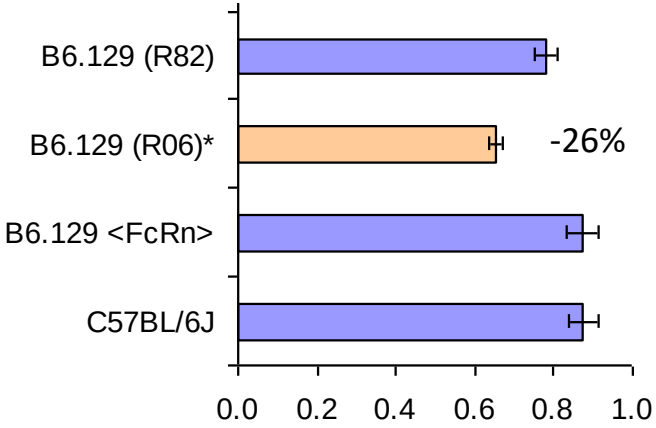
AngFq5
Vessel Area (VA)
Vessel Length (VL)
Normalized VA
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
X
Chromosome

Oca2^{p} Can Explain *AngFq5*

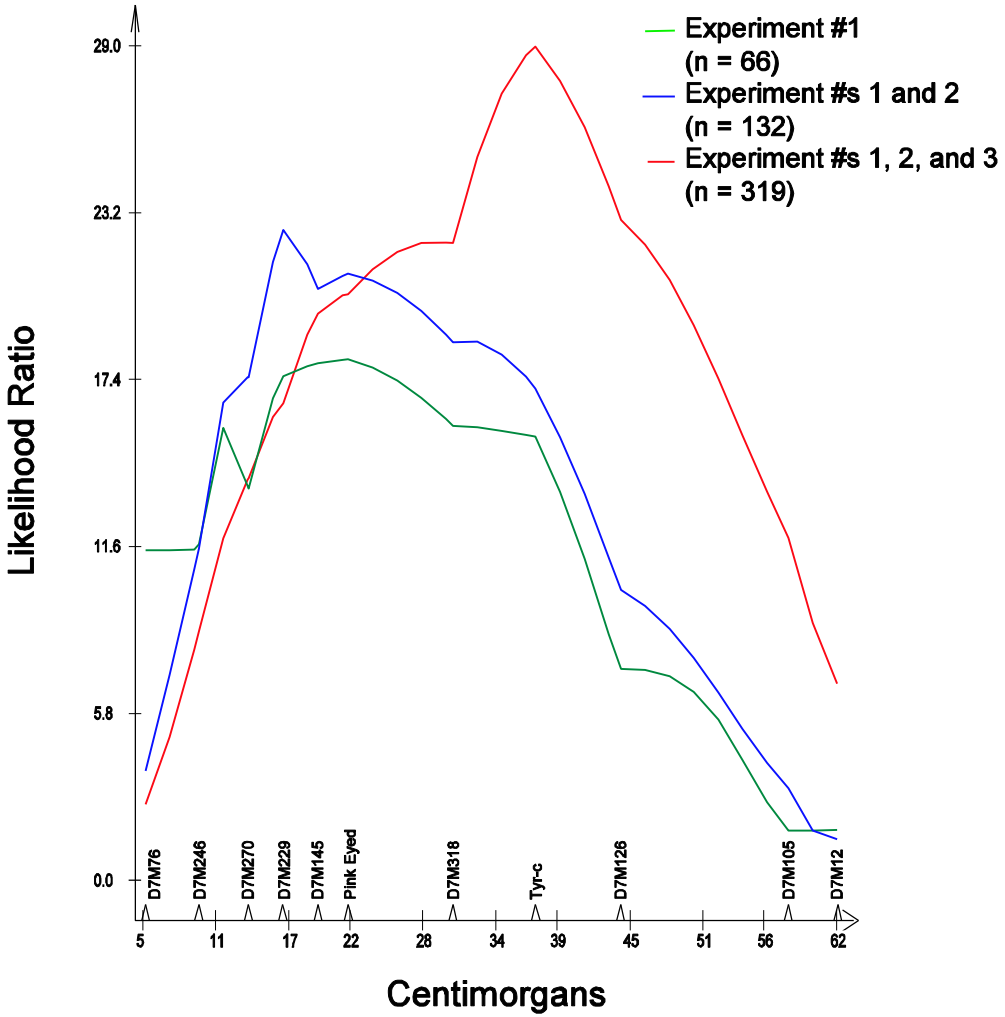




- The *pJ* allele arose independently of the classical *p* allele (in a C3H congenic, then backcrossed back to C3H).
- B6.129 strains are B6.129.7(21M) subcongenics.
- B6.129<FcRn> is a knock-out congenic.

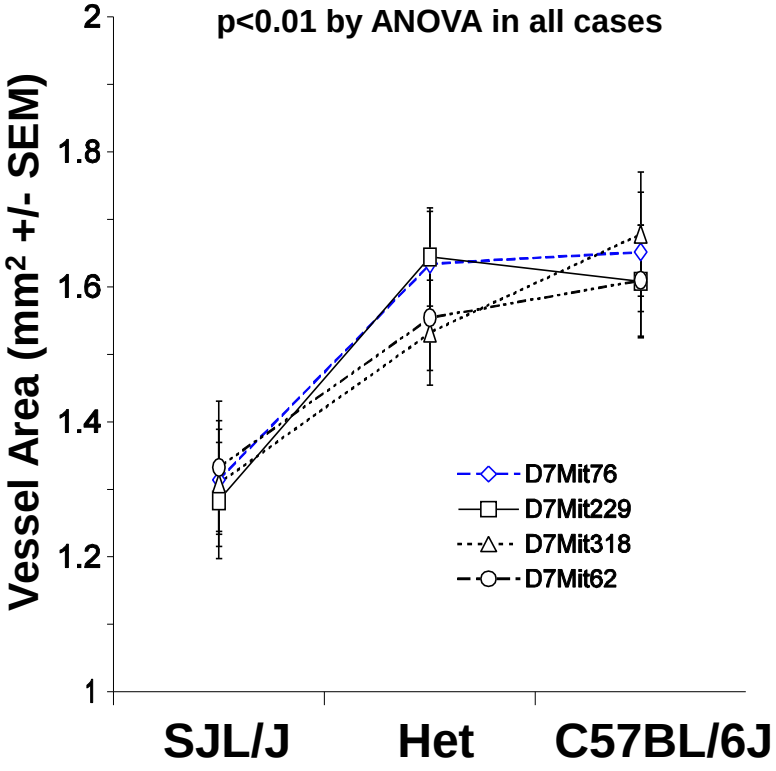
Confirmation of *AngFq5* in C57BL/6J x SJL/J

Additional F2 Animals

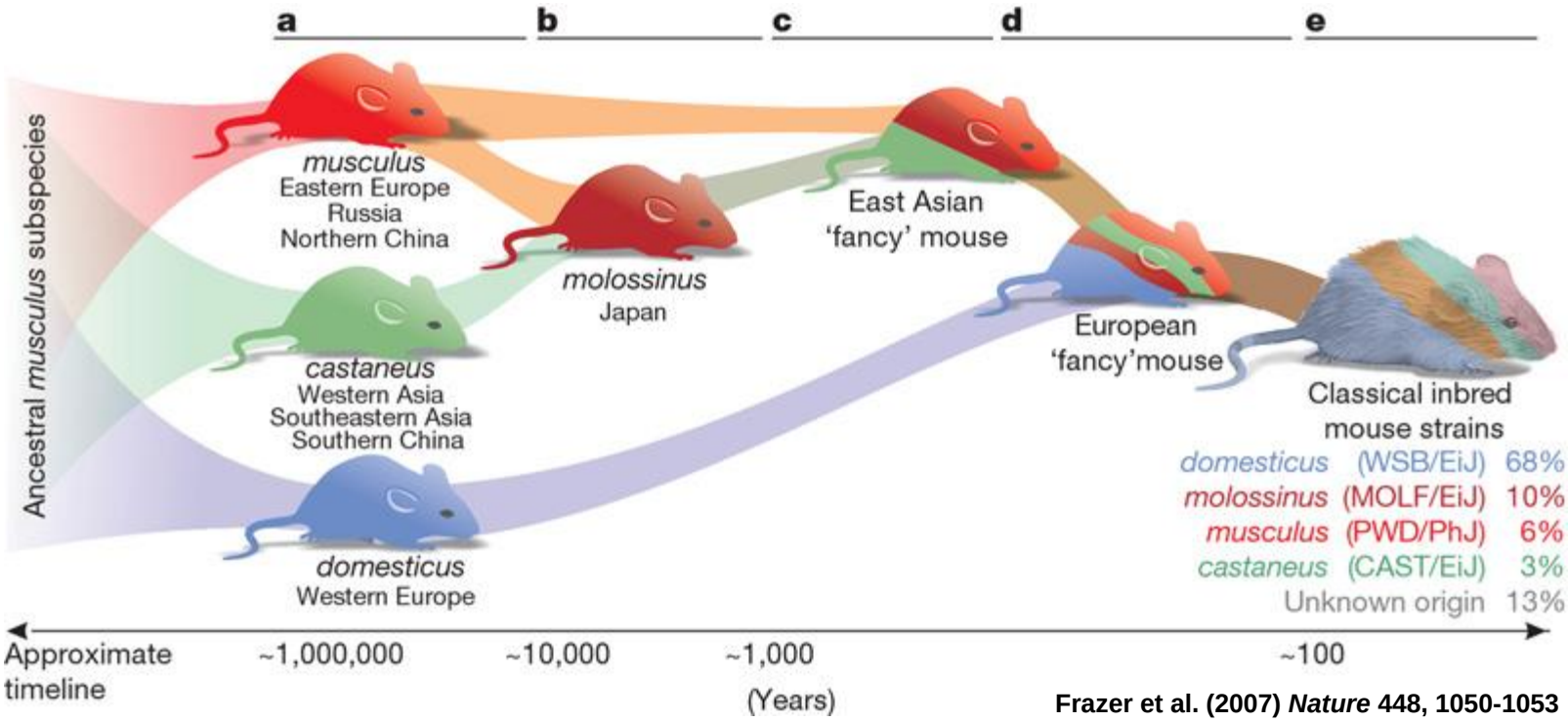


B6.SJL.7PM Congenics

Product of C57BL/6J x SJL/J F2N9F1 Cross



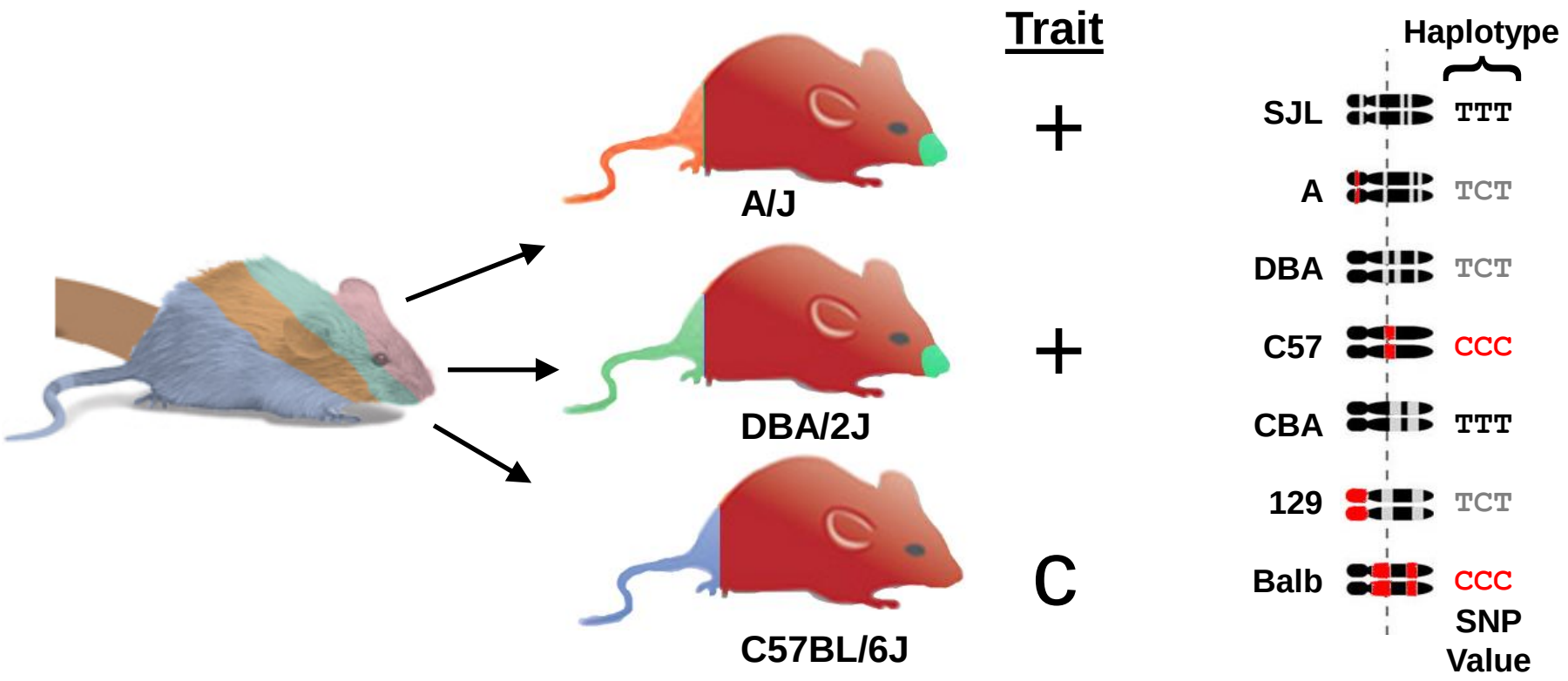
Mouse History



Frazer et al. (2007) *Nature* 448, 1050-1053

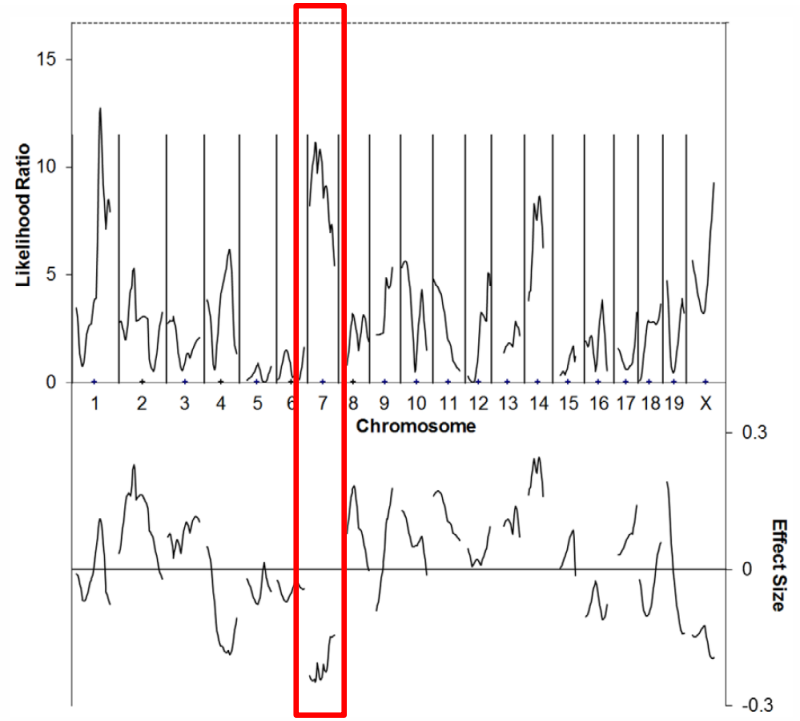
a Divergence of mouse subspecies
b *musculus* and *castaneus* hybrids form *molossinus*
c 1700s East Asian mouse fanciers breed mice for pets, coat color prized.
d Victorian breeders import 'fancy' mice and cross with local mice.
e Castle et al inbreed a limited number of ‘fancy’ mice resulting in classical strains.

Haplotype Analysis

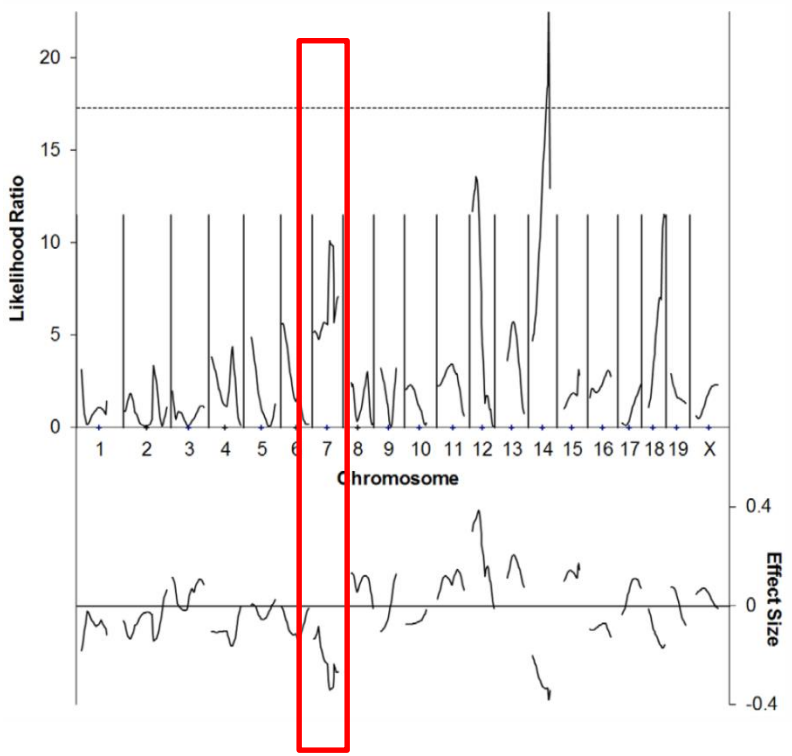


Evidence for *AngFq5* in 129 F2 Crosses (Interval Mapping)

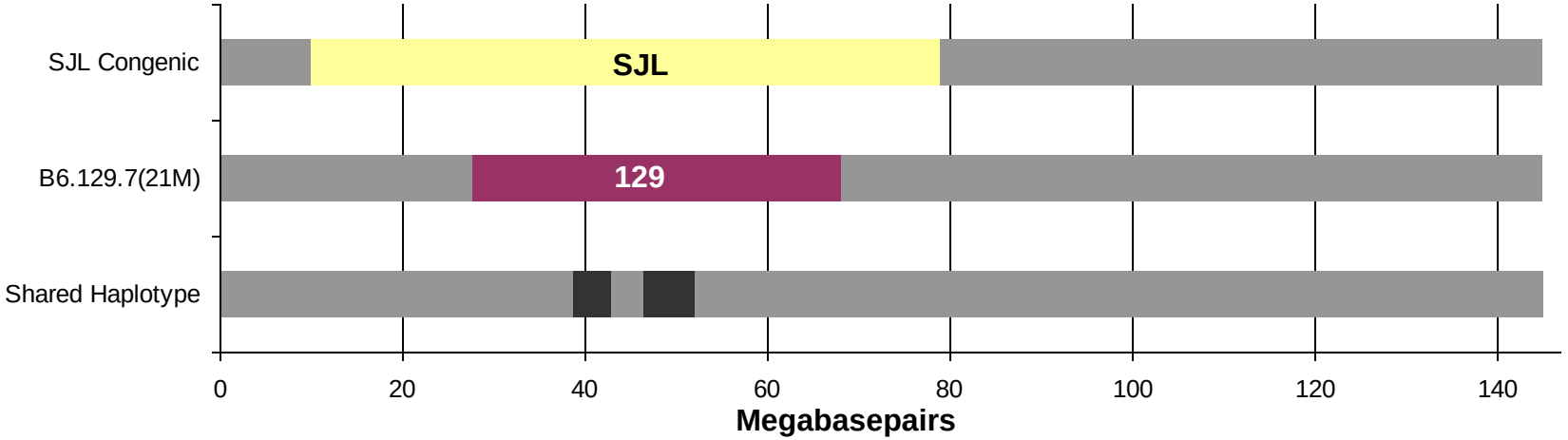
C57BL/6J x 129P1/ReJ



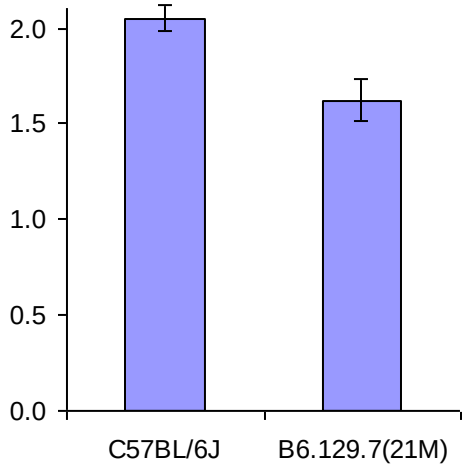
C57BL/6J x 129P3/J

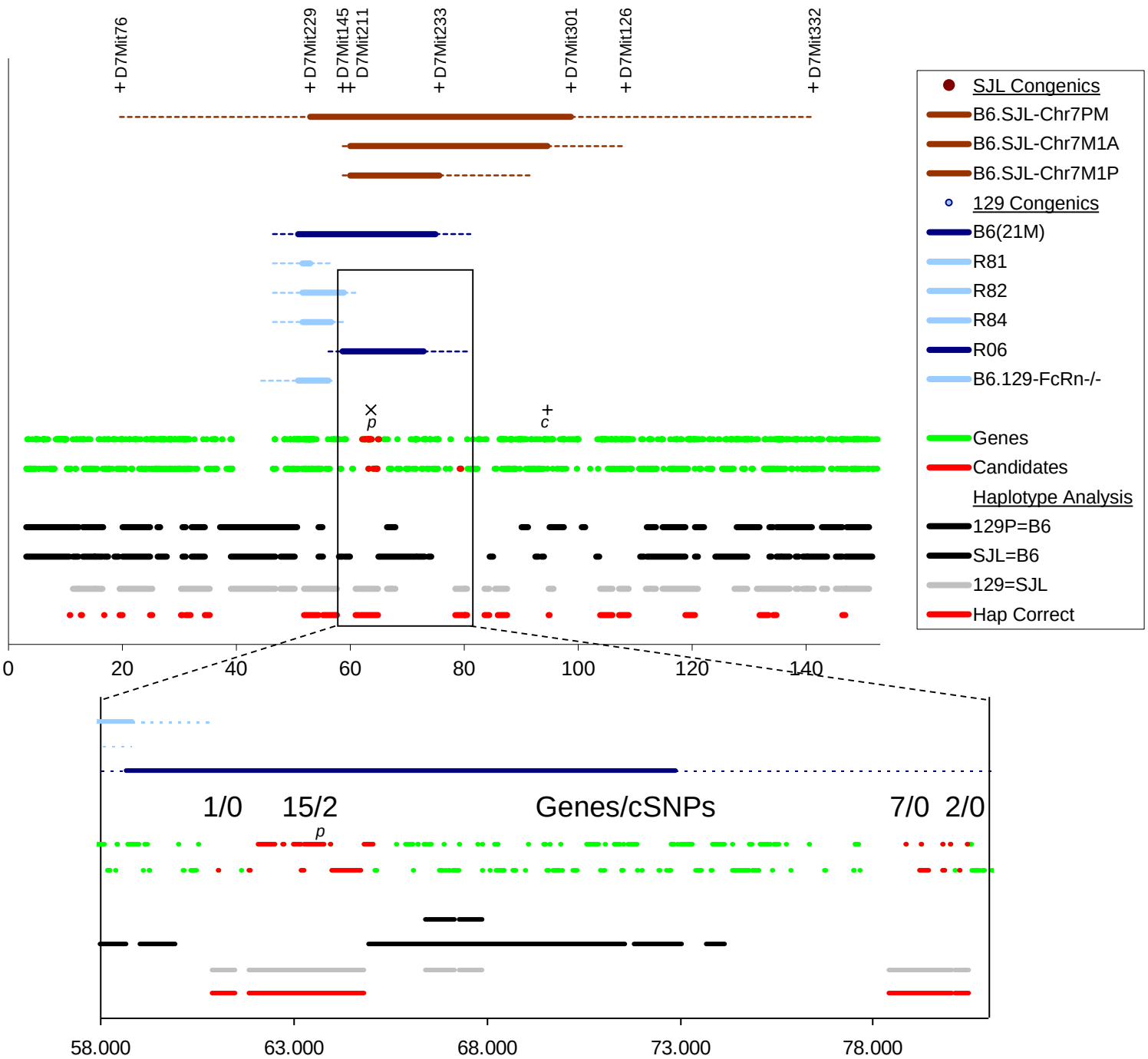


A 129 Chromosome 7 Congenics Bearing the SJL Allele at *AngFq5*

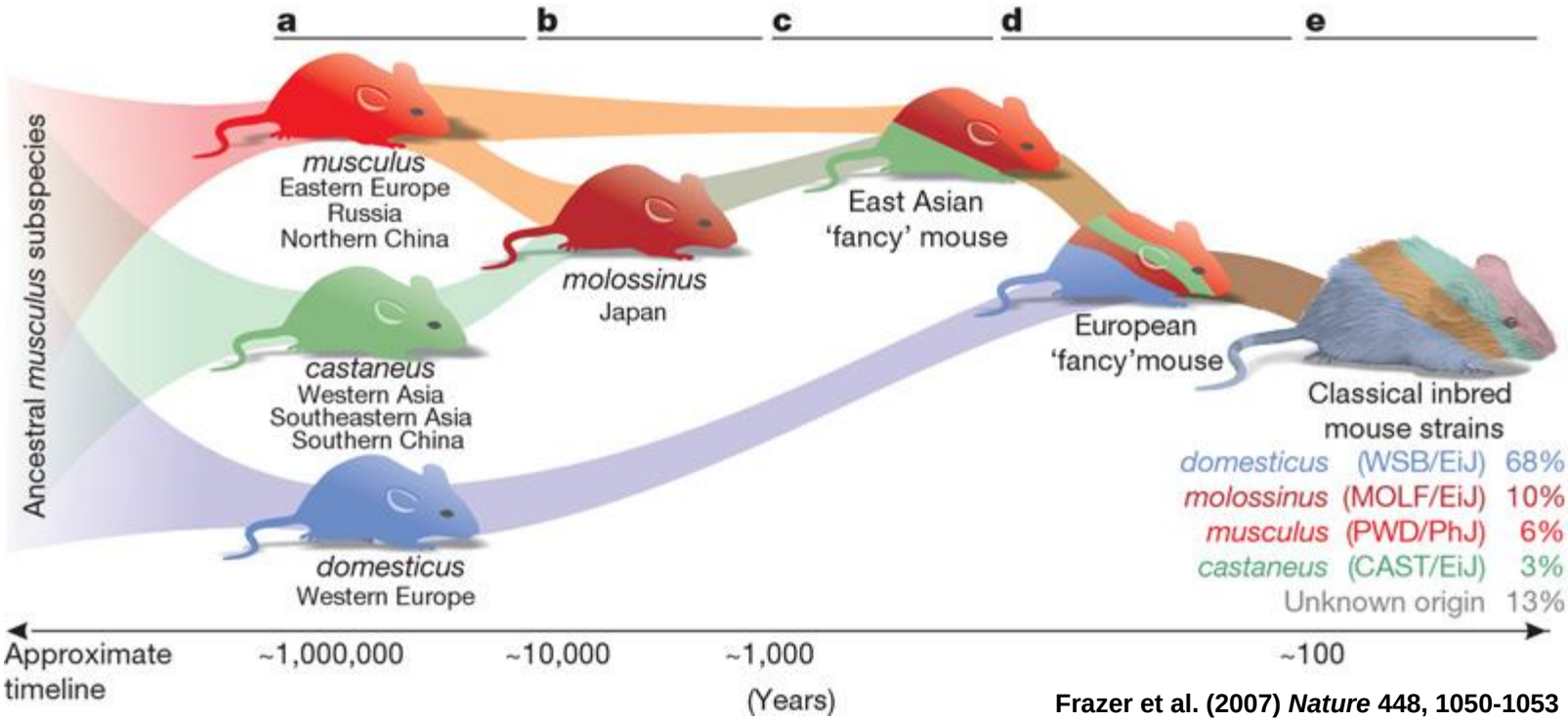


- Both 129P3/J and 129P1/ReJ F2 crosses showed linkage on Chromosome 7.
- Haplotype analysis suggests that 129 strains may bear the SJL allele of *AngFq5.*
- The B6.129.7(21M) strain bearing the region of shared haplotype (Jackson Labs) shows a decrease in angiogenesis consistant with *AngFq5.*




D7Mit76
D7Mit229
D7Mit145
D7Mit211
D7Mit233
D7Mit301
D7Mit126
D7Mit332
p
c
0
20
40
60
80
100
120
140
SJL Congenics
B6.SJL-Chr7PM
B6.SJL-Chr7M1A
B6.SJL-Chr7M1P
129 Congenics
B6(21M)
R81
R82
R84
R06
B6.129-FcRn-/-
Genes
Candidates
Haplotype Analysis
129P=B6
SJL=B6
129=SJL
Hap Correct
1/0
15/2
Genes/cSNPs
7/0
2/0
58.000
63.000
68.000
73.000
78.000

Mouse History



Frazer et al. (2007) *Nature* 448, 1050-1053

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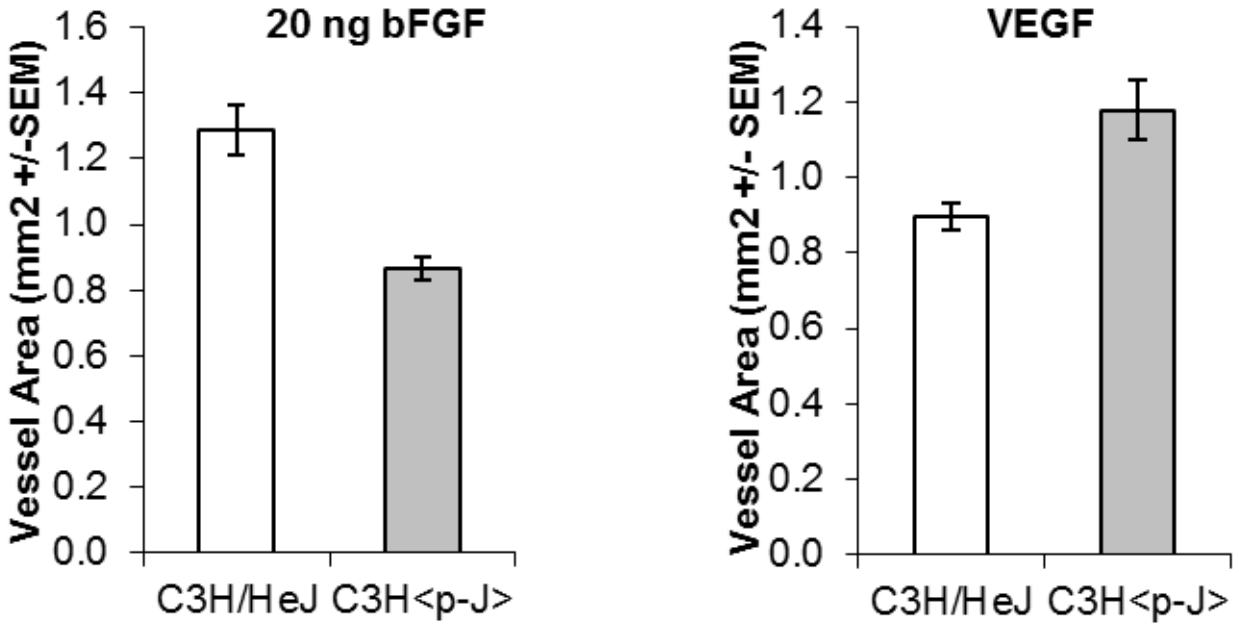
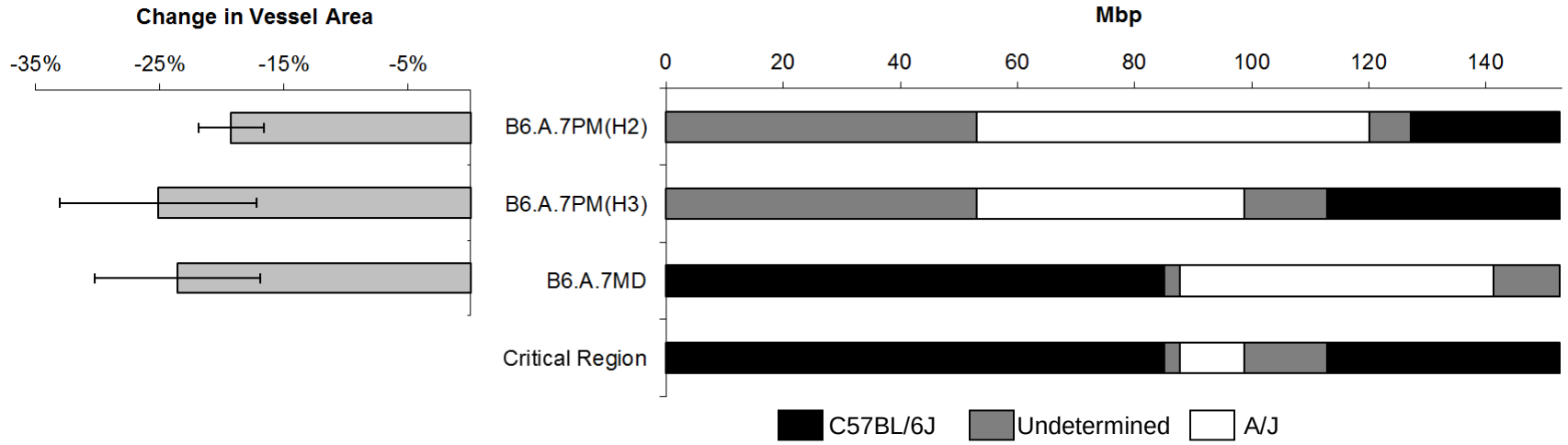
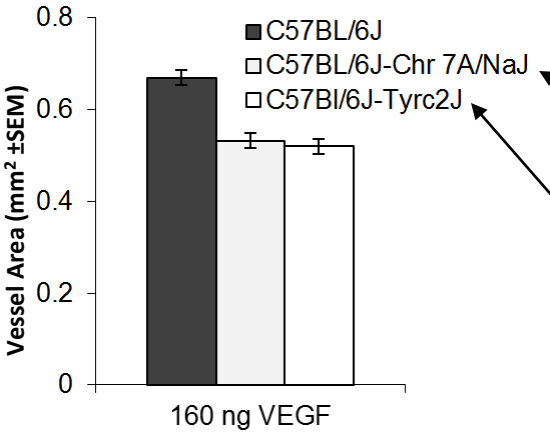
d Victorian breeders import 'fancy' mice and cross with local mice.
e Castle et al inbreed a limited number of ‘fancy’ mice resulting in classical strains.

A Third *Oca2* Allele Confirms that Pink-eyed Dilution Mutations Affect Angiogenesis



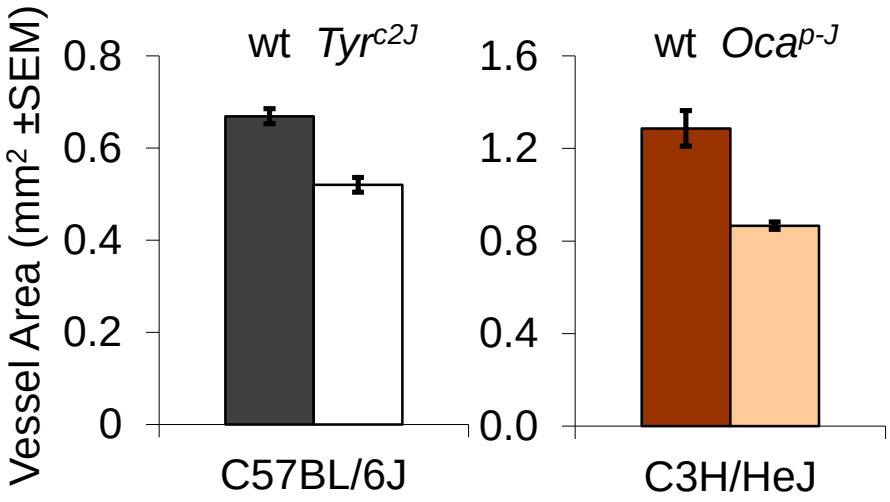
The tyrosinase albino mutation can explain *AngVq4*.



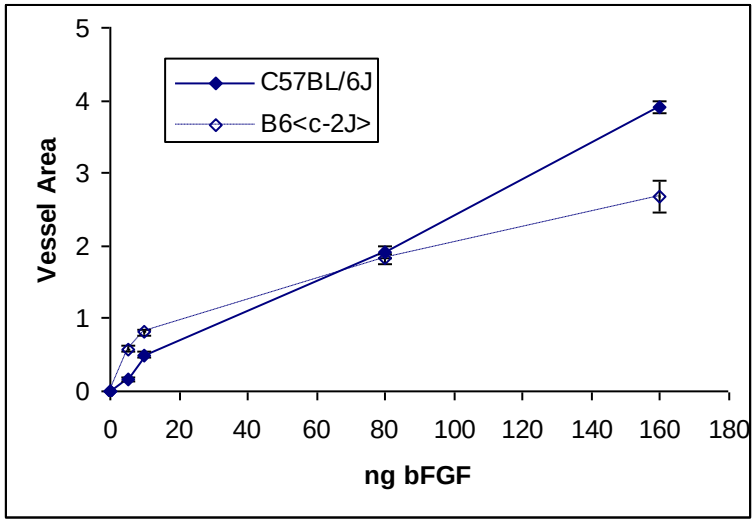


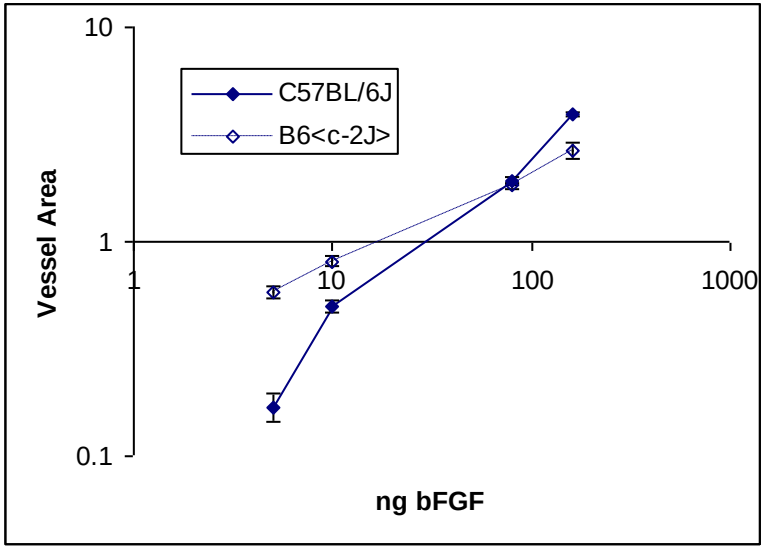
-19th Century—Tyr^{c} (C85S) containing Asian pet mice brought to Europe.
-Early 20th Century—A/J (albino) strain established with much of Chr. 7 from Asian mice (many additional differences with C57BL/6J).

-1970—Tyr$^{c\text{-}2J}$ (R77L) arose spontaneously in C57 background.

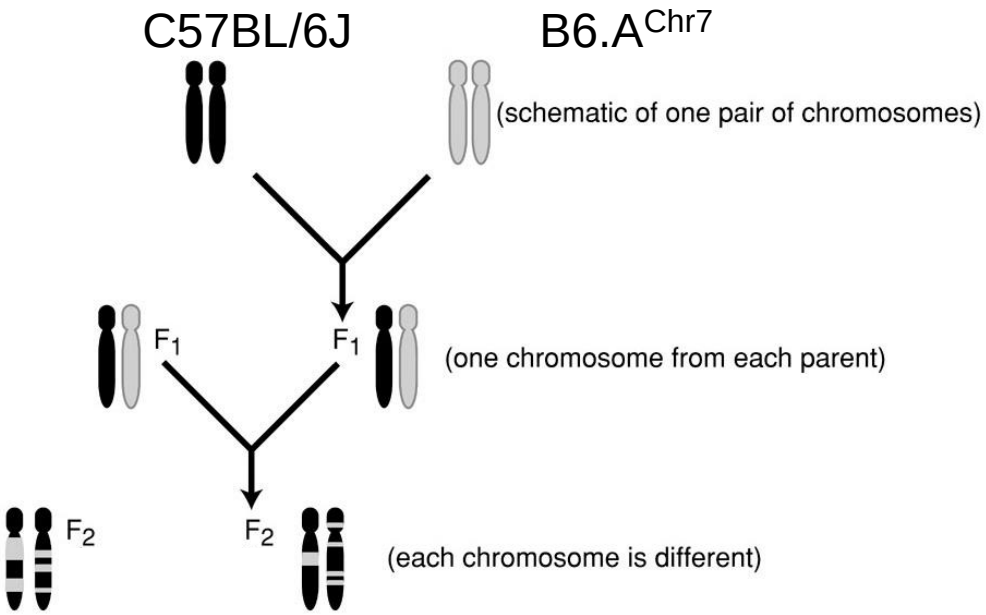

Vessel Area (mm² ±SEM)
0.8
0.6
0.4
0.2
0
wt
Tyrc2J
C57BL/6J
1.6
1.2
0.8
0.4
0.0
wt
Ocap-J
C3H/HeJ

Difference in Angiogenic Response to bFGF Varies by Dose





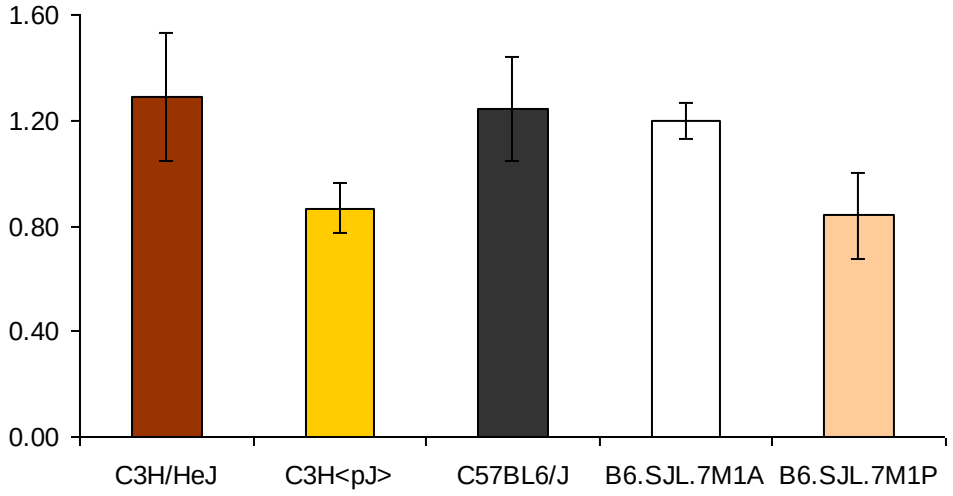
F2 Intercross



Marker	b0	b1	F	%Var	P
D7Mit229	0.787	-0.220	5.265	8.88%	0.025
D7Mit318	0.781	-0.248	8.825	14.05%	0.004
D7Mit62	0.780	-0.239	8.094	13.04%	0.006
Tyr	0.767	-0.234	8.863	14.10%	0.004
D7Mit301	0.773	-0.210	8.600	13.74%	0.005
D7Mit238	0.770	-0.201	8.048	12.97%	0.006
D7Mit186	0.761	-0.156	4.514	7.71%	0.037
D7Mit332	0.763	-0.177	6.884	11.31%	0.011

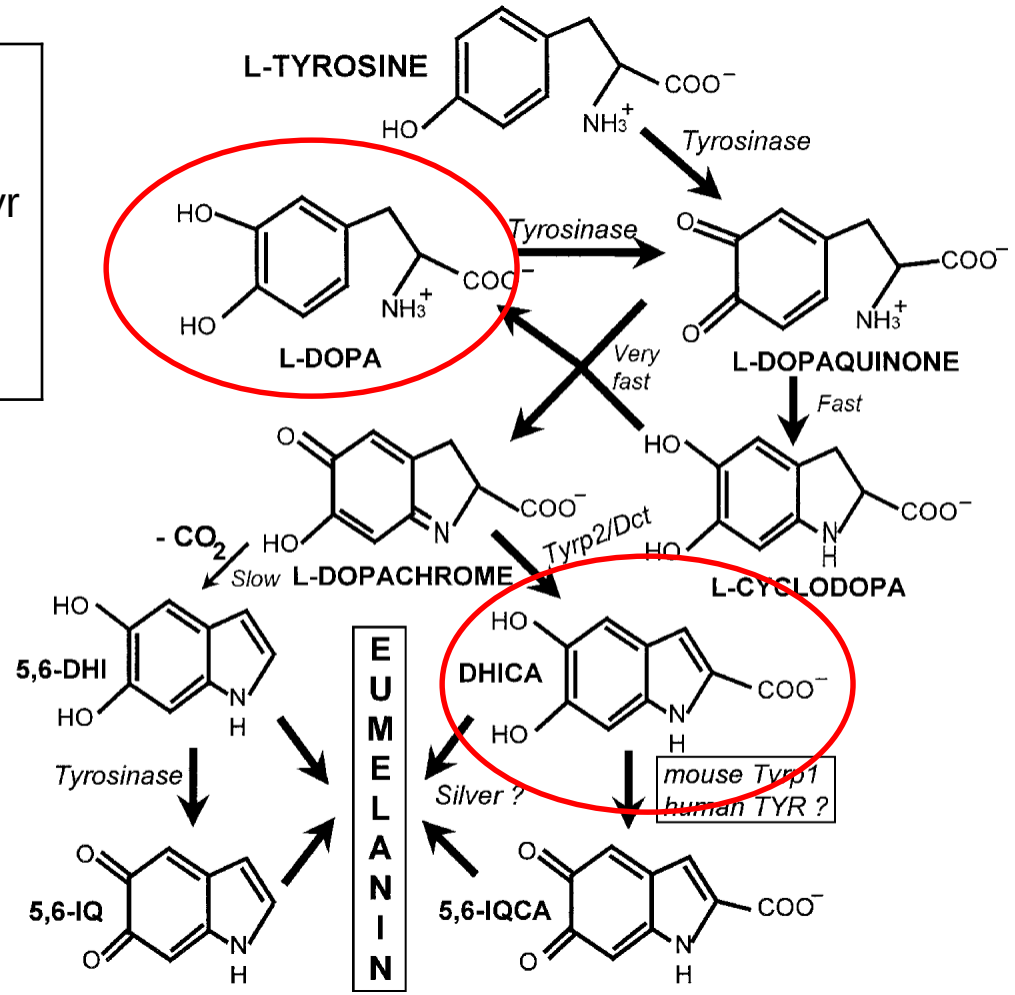
Epistasis Between *p* and *c* in Angiogenesis Conforms to Pigment-based Expectations

- Tyr^{c2J} Doesn't affect bFGF-induced Angiogenesis (80 ng).
- *ccpp* Animals are Albino in Color.
- B6.SJL.7M1A animals are *ccpp*.
- B6.SJL.7M1P animals are *CCpp*.
- Historical Significance (Haldane 1915)



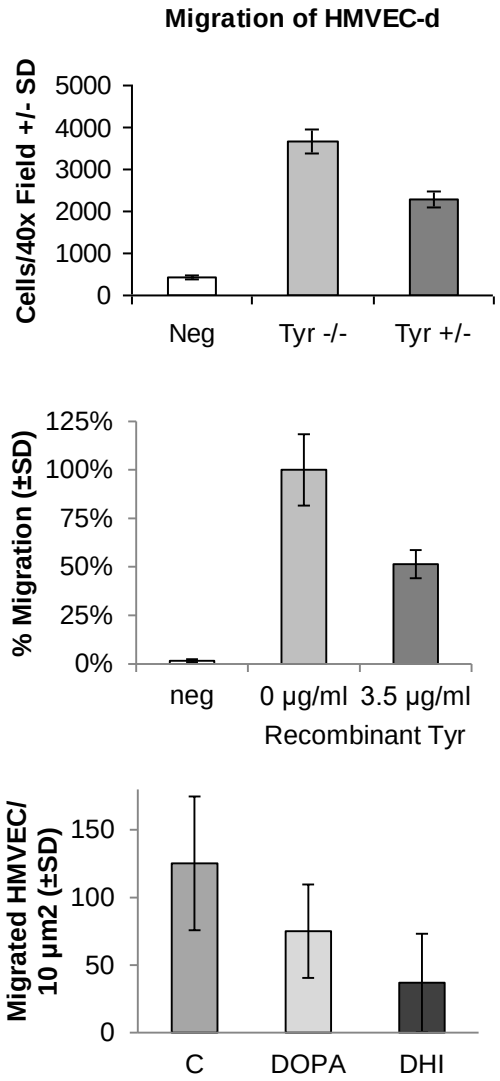
Melanogenesis

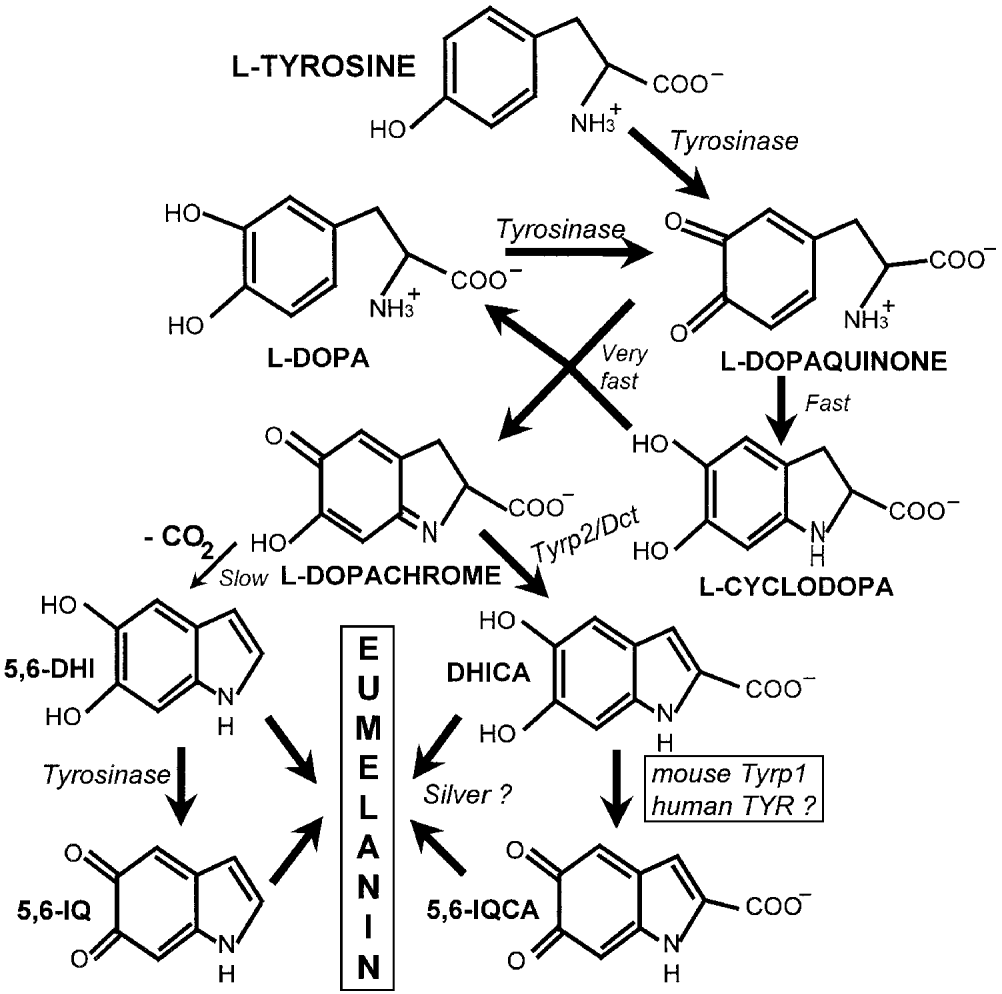
	Corneal Angiogenesis		DHICA,
	bFGF	VEGF	Plasma Tyr
Oca2^{p}	↓	↑	↑
Tyr^{c}	↑	↓	↓



Olivares et al, Biochem. J. (2001)

Tyr Products Affect HMVECd Migration



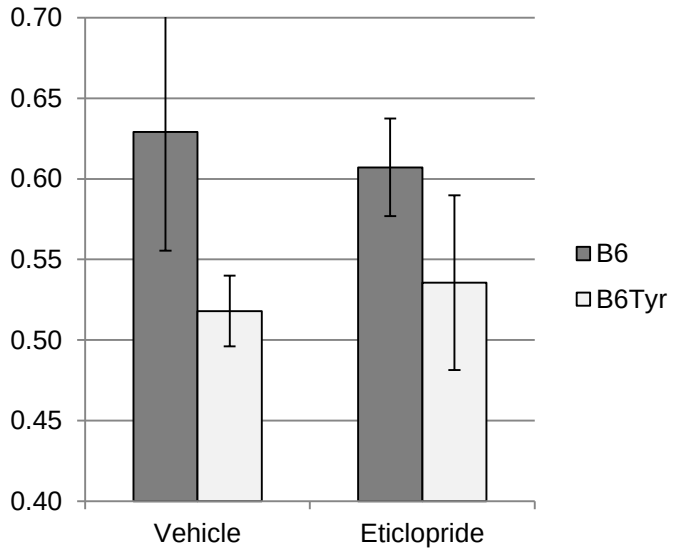


Olivares et al, Biochem. J. (2001)

Attempts to Identify Tyrosinase Products that Modulate Corneal Angiogenesis

Eticlopride (Dopamine D2 Antagonist)

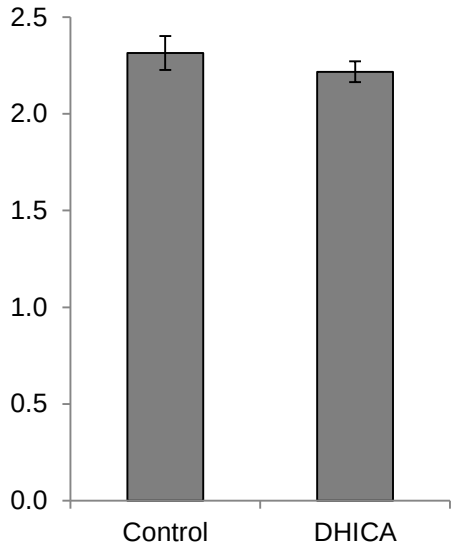
10 ng bFGF



10mg/kg/day/IP

DHICA (1st stable product of tyrosinase)

80 ng bFGF



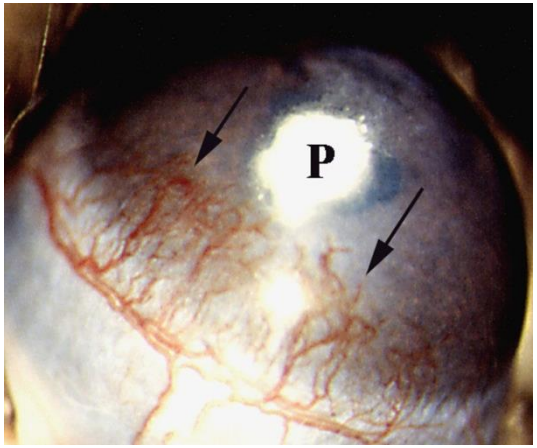
10mg/kg/day/IP

Should try both of these with VEGF

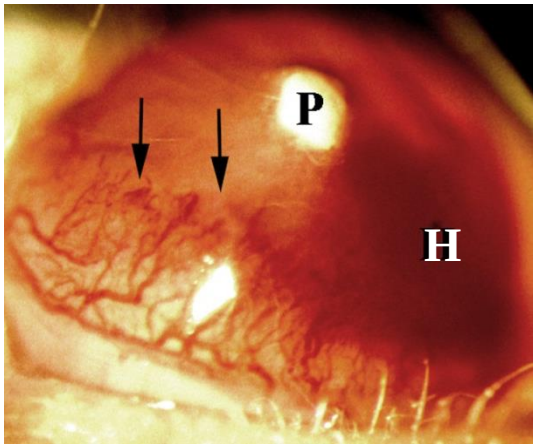
Hyphema Formation Secondary to Iris Neovacularization Induced by Corneal Pellets in Albino C57 Mice

bFGF Pellets

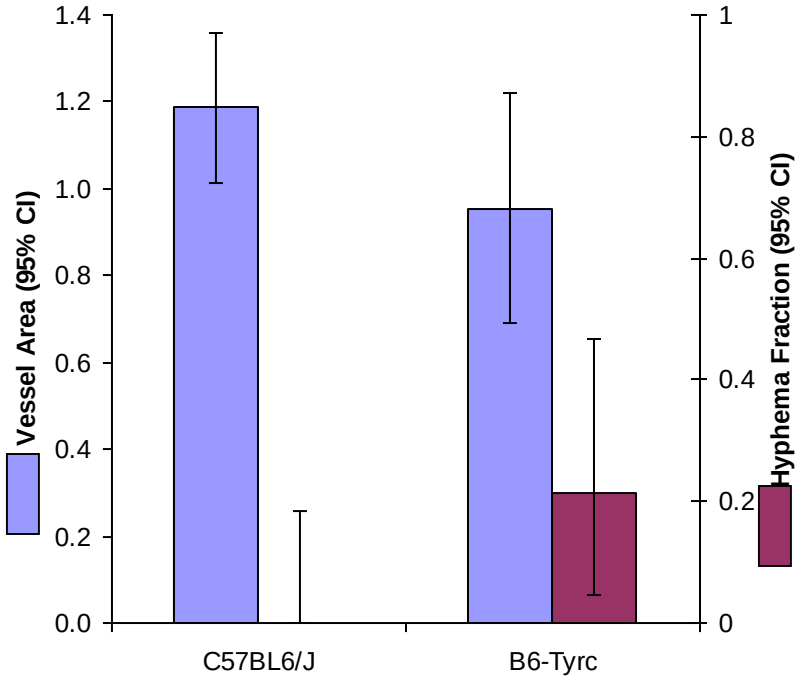
C57BL/6J



C57BL/6J *Tyr* $^{c-2J}$



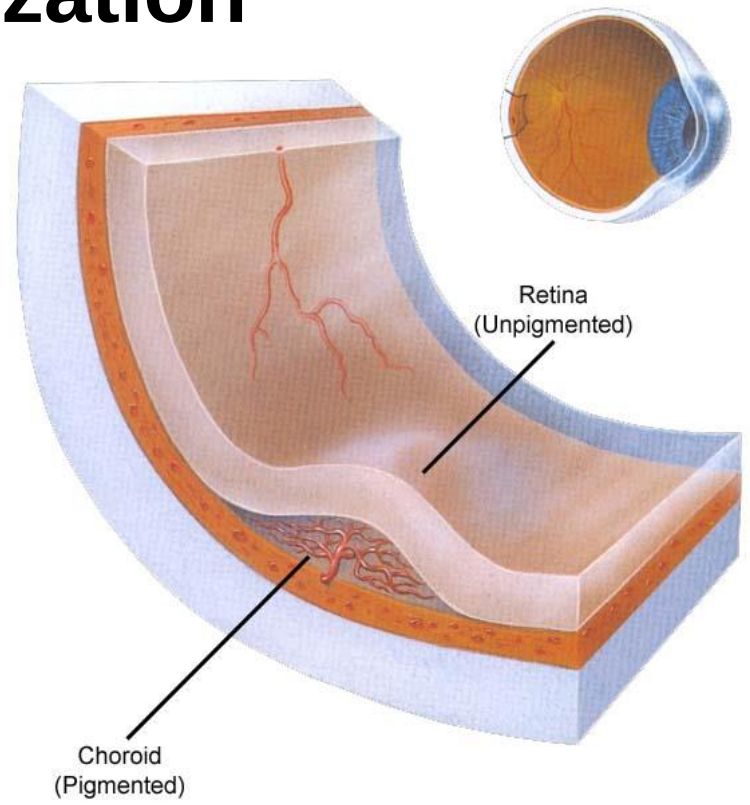
4x (800 ng) VEGF Pellets



Rogers, et al *Angiogenesis* 2013

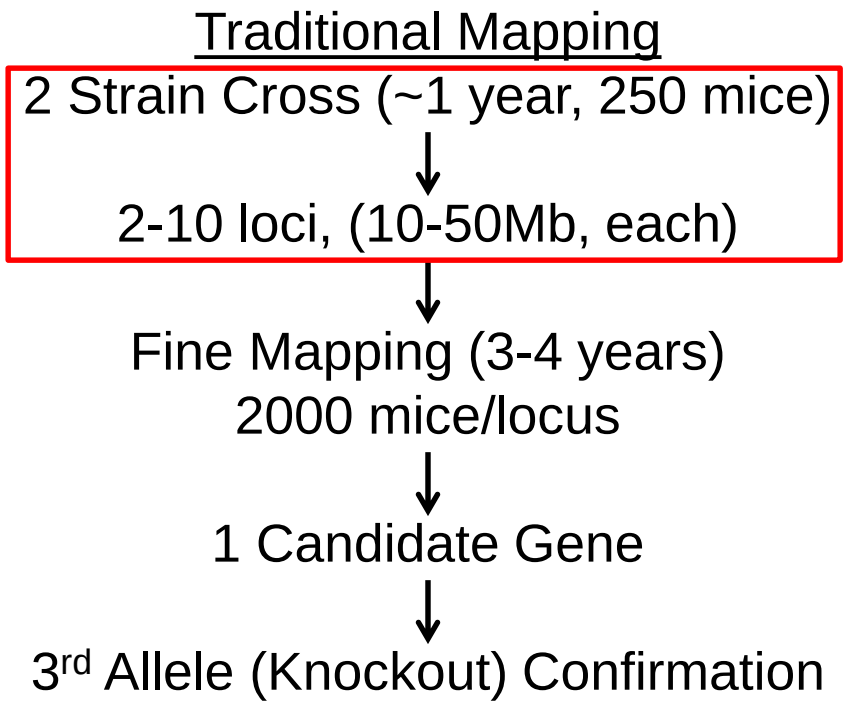
The Effect of Albino Mutations on Eye Neovascularization

	Cornea (no Pigment)	Iris (Pigment)
bFGF	↑↓	↑
VEGF	↓	↑



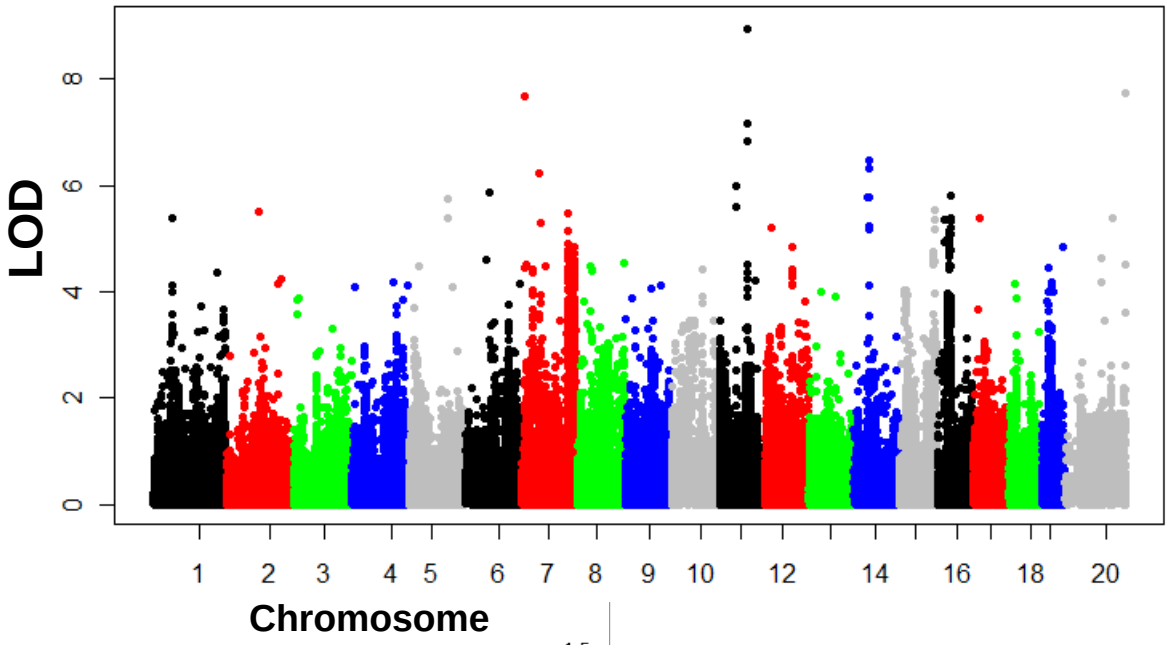
- Macular Degeneration (adjacent to pigmented choroid)—Less severe in African Americans.
- Diabetic Retinopathy (unpigmented tissue)—much more severe in African Americans (currently attributed to poorer blood sugar control).

Can We Speed this Up?

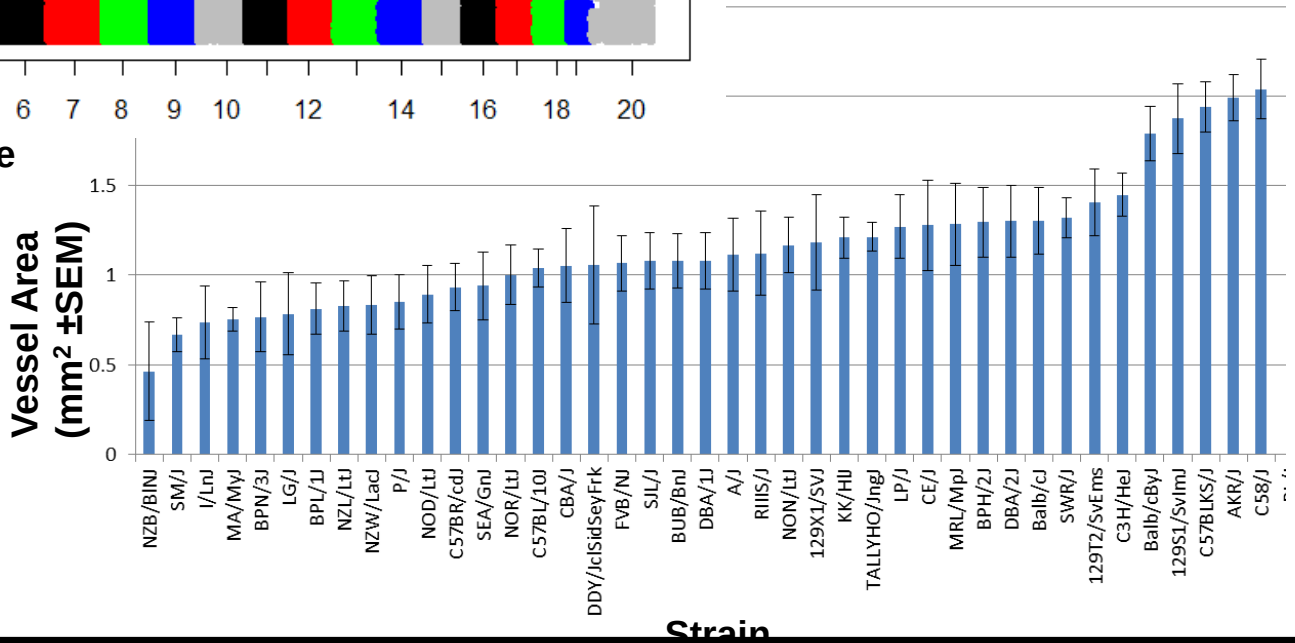


- Samples Limited Genetic Diversity
- Generates Large Regions that Become Progressively Harder to Map (Crossovers Become Rare and are not Randomly Distributed)

Genome-wide Association (20ng bFGF)



$$y_i = \mu + bx_i + Zu + e_i$$

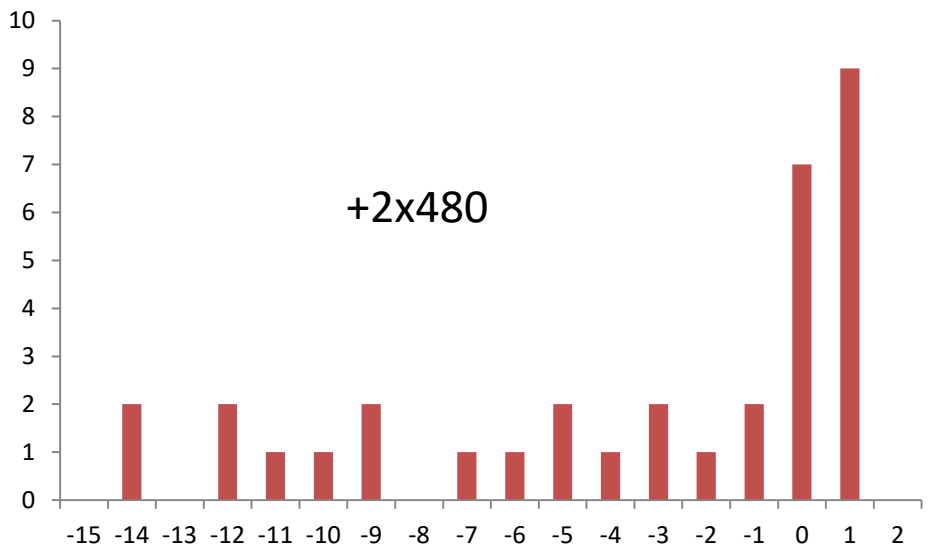


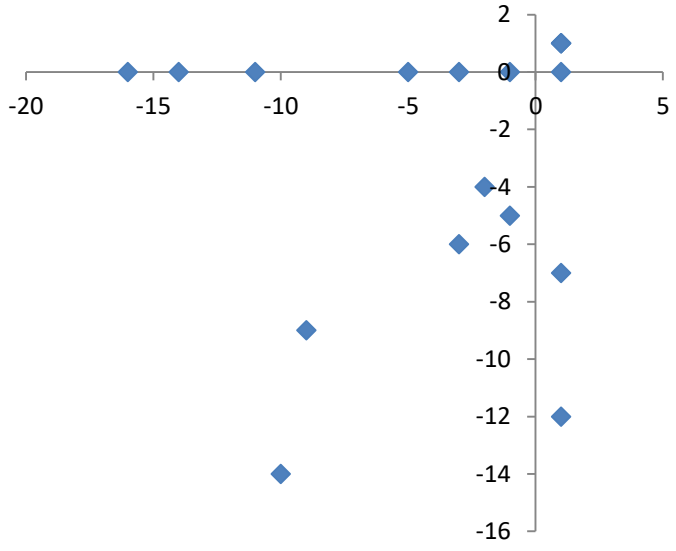
Target Gene Selection and sgRNA Design

- Pigment Production Genes
  - No non-pigment annotations
  - One per chromosome

- sgRNA Design
  - No validated techniques predict activity in mouse embryos
  - Used CHOPCHOP (chopchop.rc.fas.harvard.edu) to select unique sites
  - Predicted activity using the GPP Web Portal @ the Broad (Doench 2014)
  - For each gene, selected two sites
    - a) near the beginning of the gene with
    - b) no 1 or 2 base mismatches in the genome, and
    - c) an activity score >0.6
  - Designed 5 primers for each site (2x PCR, 2x coding, 1x IVT), truncating as possible (tru-sgRNAs have equal activity, less off-target).

Distribution of Alleles in Mice with at Least One Targeting Event

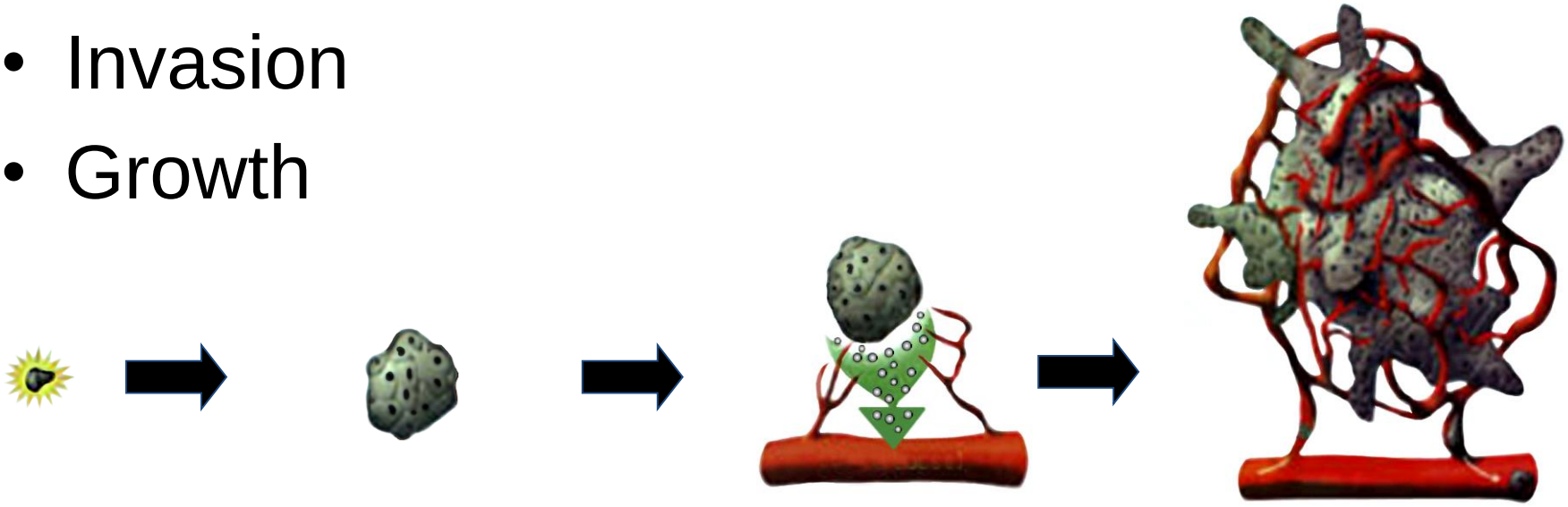




(How) Do Differences in Host Neovascular Response Affect Tumor Growth?

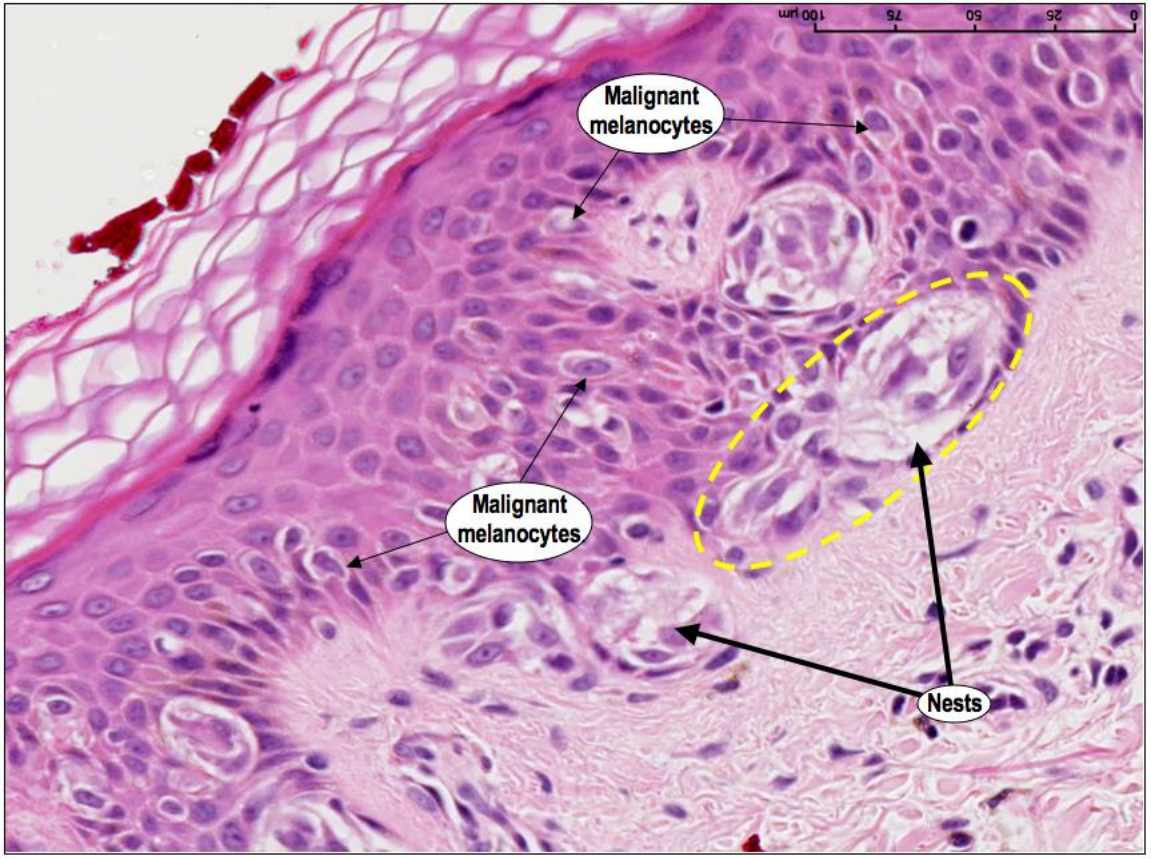
Key Features of Cancer

- Proliferation
- Dysplasia/ Neoplasia
- Invasion
- Growth



Melanoma (Horizontal Phase)

- ✓Proliferation
- ✓Dysplasia/ Neoplasia
- ✓Invasion
- Growth



https://micro2tele.files.wordpress.com/2014/01/1-malignant-melanocytes.png

Dormant *in situ* cancers (in people who died of trauma).

---

1. In autopsies of women from 40 to 50 years old,
   39% have small carcinomas in their breasts.
   +/+-But, cancer is diagnosed in only **1%** of women
   in this age range.

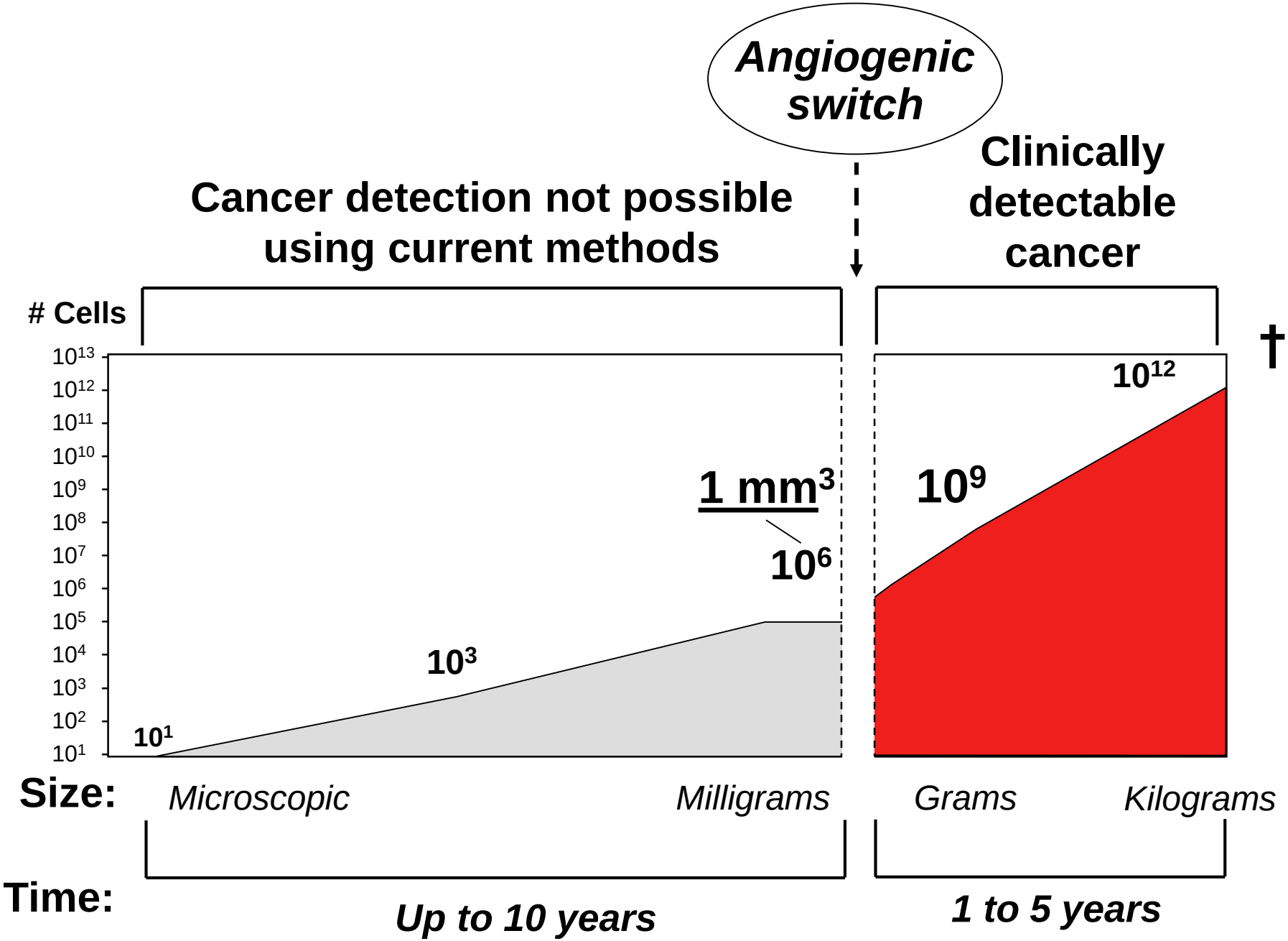
---

2. In men from age 60 to 70,
   46% have small prostate tumors.
   +/+-But, only **1%** are diagnosed clinically in this age range.

---

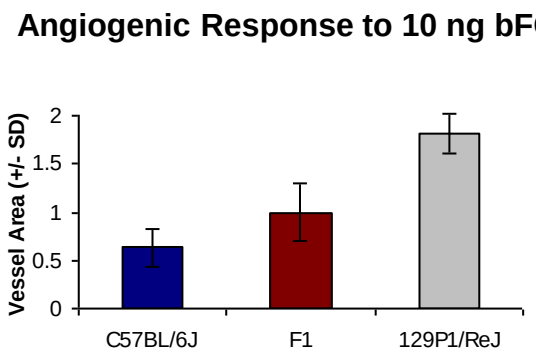
3. Autopsies of people from age 50 to 70 show that
   virtually all have small thyroid tumors.
   +/+-But, thyroid cancer is diagnosed in only
   0.1% of people in this age group.

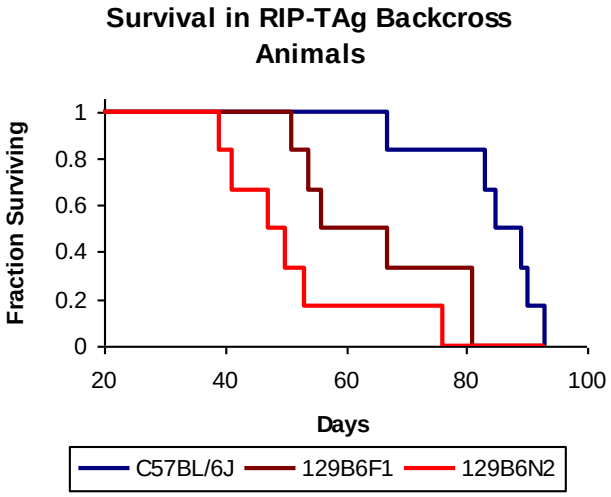
Black and Welch, *N.E.J.M.* 328: 1237-1243, 1993

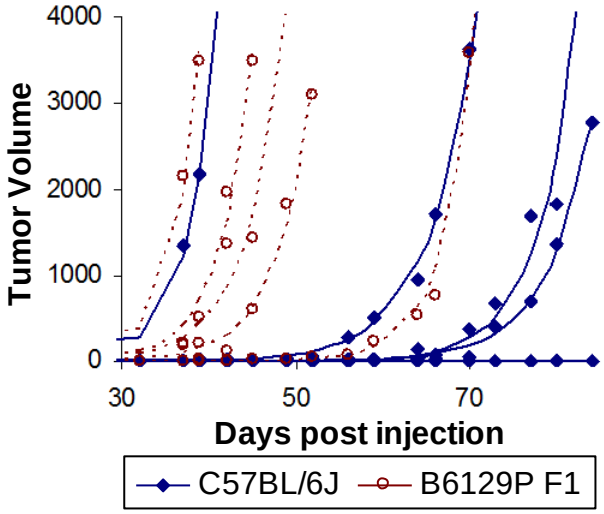


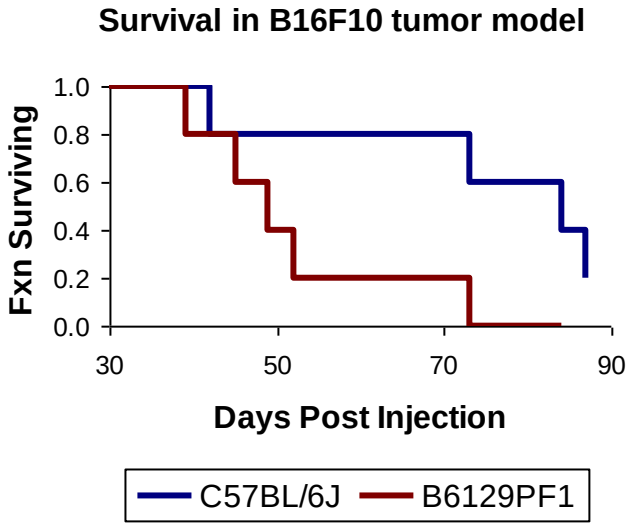
MW Retsky, et al Cancer Res. 47:4982, 1987.
J. Heymach, unpublished 2005.

Increased Angiogenic Responsiveness and Decreased Survival in Mouse Models



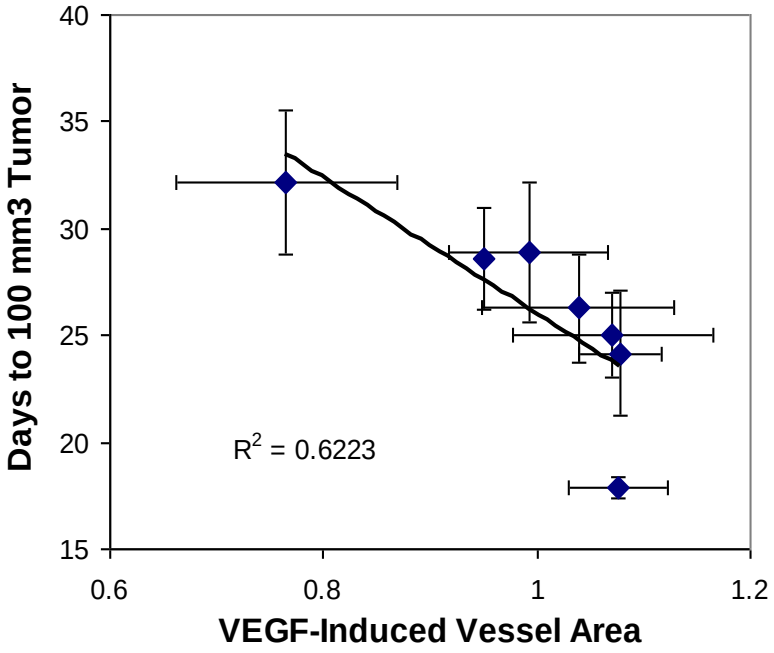






~3x105 B16F10 melanoma cells injected. Tumor free survival of the same mice (p=0.03).

Tumor Latency Correlates to Angiogenic Responsiveness in BXD Recombinant-inbred Mice



- ~600,000 B16F10 melanoma cells were injected subcutaneously into 5 each of 7 BXD mouse strains of *H2b* haplotype.
- Perpendicular tumor diameters were measured twice weekly and tumor volume was calculated using the formula V=0.52*l*w2.
- The day that a tumor exceeded 100 mm3 was interpolated from the measurement immediately before and after that point using a log-linear plot.
- No strains that exhibited spontaneous regression of tumor are included.

Relevance to Human Health?

- Susceptibility to melanoma varies >20-fold among different populations.
- Prominent among within-population genetic polymorphisms that affect melanoma susceptibility are those in pigment production genes such as: ASIP, MC1R, OCA2, SLC45A2, TYR.
- What about UV susceptibility as mechanism for these alleles?

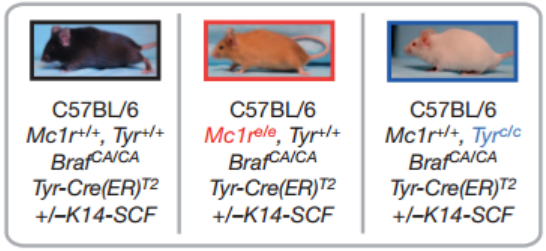
LETTER

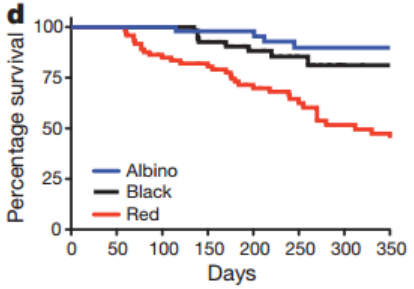
doi:10.1038/nature11624

An ultraviolet-radiation-independent pathway to melanoma carcinogenesis in the red hair/fair skin background

Devarati Mitra[1], Xi Luo[2], Ann Morgan[1], Jin Wang[3], Mai P. Hoang[4], Jennifer Lo[1], Candace R. Guerrero[3], Jochen K. Lennerz[5], Martin C. Mihm[4], Jennifer A. Wargo[6], Kathleen C. Robinson[1], Suprabha P. Devi[1], Jillian C. Vanover[7], John A. D'Orazio[7], Martin McMahon[8], Marcus W. Bosenberg[9], Kevin M. Haigis[2], Daniel A. Haber[2], Yinsheng Wang[3] & David E. Fisher[1]

Nature (2012) 491:449-53





How Might this Improve Patient Management?

Individuals at High Risk of Developing a New Tumor

- Cancer Patients
  - Metastases
  - Second Primary Neoplasms
    (18% of Diagnoses, only 3% at Nearby Site)
- High Risk Behaviors (Smoking, etc.)
- Individuals Bearing Risk Alleles (e.g. BRCA1/2, etc.)

Novel Targets for Antiangiogenic Therapy

- Not Dependent on Known Pathways
- Already Known that Modulation is Compatible with Life (Anticipate Reduced Side-effects)