The discovery of new targets for treatment of inflammatory disorders

Brussels

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Biological Activity of Cachectin

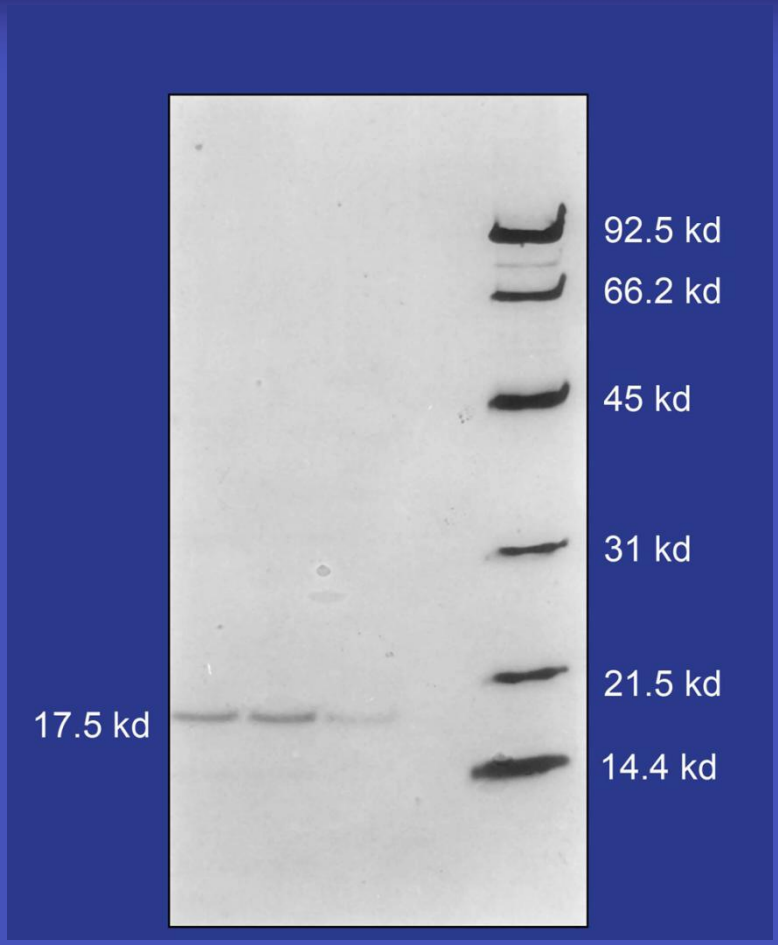
- Presumed important for wasting in chronic disease, cachectin was secreted by macrophages in response to LPS.
- Defined by its ability to shut off the synthesis of adipocyte lipoprotein lipase, an enzyme needed for the uptake and storage of plasma triglycerides.

1983

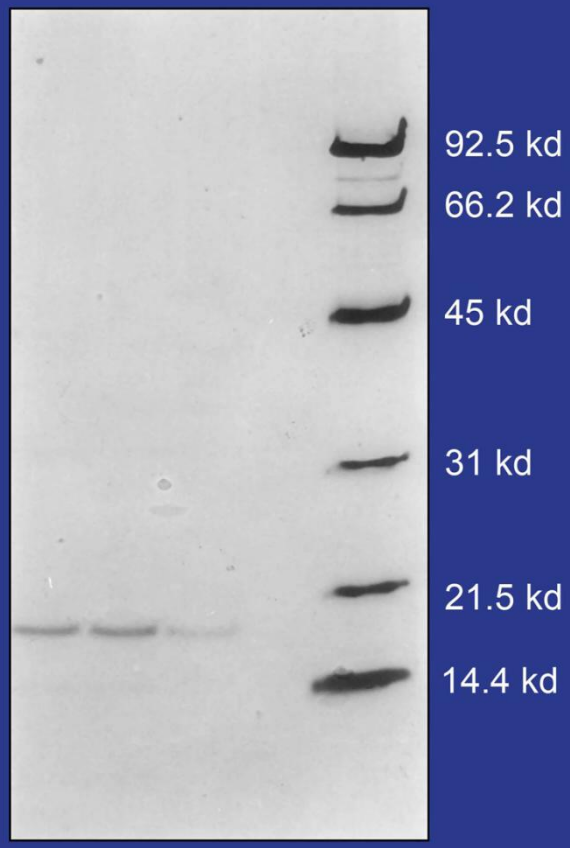
Isolation of mouse cachectin

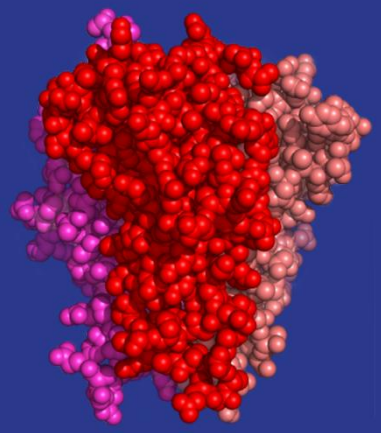
- Pressure dialysis of medium from ~500 10 cm plates of LPS-activated RAW 264.7 cells (early harvest)
- ConA sepharose chromatography
- Isoelectric focusing in a glycerol gradient
- Preparative native gel electrophoresis
- Preparative SDS gel electrophoresis
- Yielded microgram quantities of an apparently pure 17.5 kD protein with approximately 2% yield of initial biological activity (prior to denaturing gel electrophoresis).
- Cachectin comprised 1-2% of the protein secreted by RAW 264.7 cells during the first two hours following LPS activation.

1984



Leu-Arg-Ser-Ser-Ser-Glu-Asn-Ser-Ser-Asp-Pro-Pro-Val-Ala-?-Val-Val-Ala-Asn…





TNF

Cachectin = Mouse tumor necrosis factor

(mouse CACH)

H_2N LEU-ARG-SER-SER-SER-GLU-ASN-SER-SER-ASP-PRO-PRO-VAL-ALA- ? -VAL-VAL-ALA-ASN…

H_2N VAL-ARG-SER-SER-SER-ARG-THR-PRO-SER-ASP-LYS-PRO-VAL-ALA-HIS-VAL-VAL-ALA-ASN…

(human TNF)

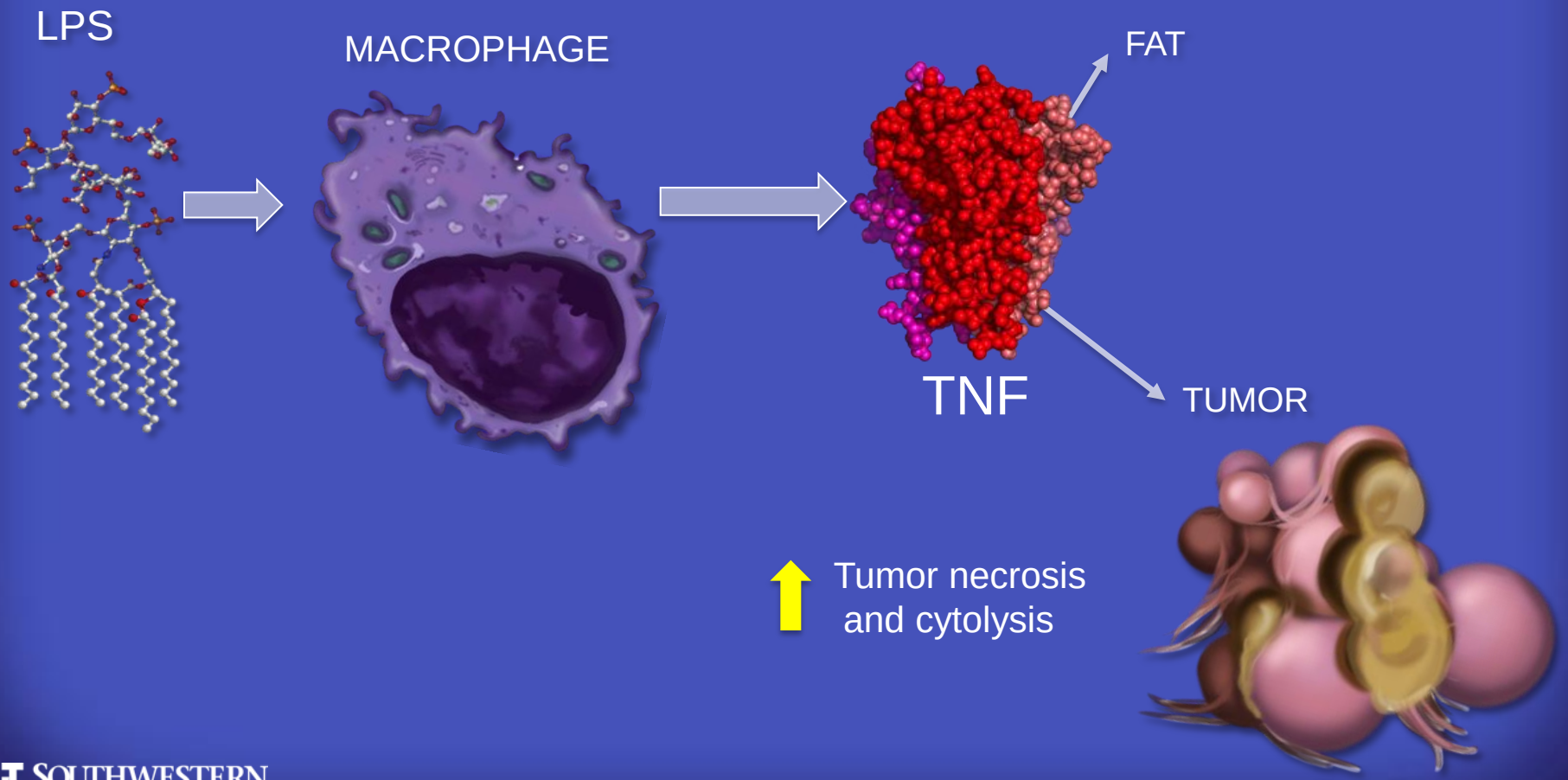
1 µg of cachectin had 10^8 U of TNF activity

1985

TNF mediated diverse effects of LPS. This raised the question: might TNF mediate *all* effects of LPS, including the lethal effect?

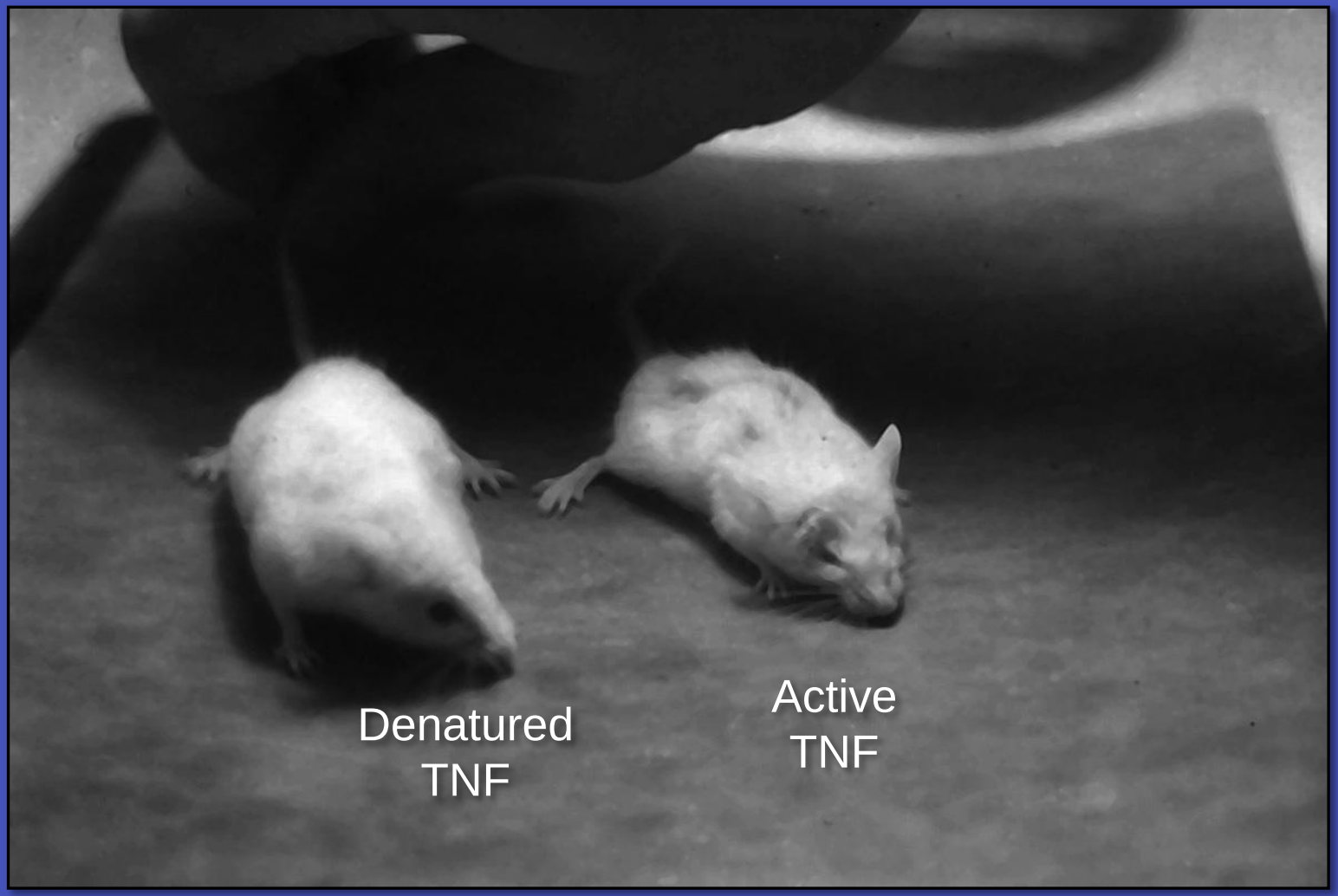
↓ Triglyceride synthesis, LPL, FAS

↑ AcCoA carboxylase, glycerol release



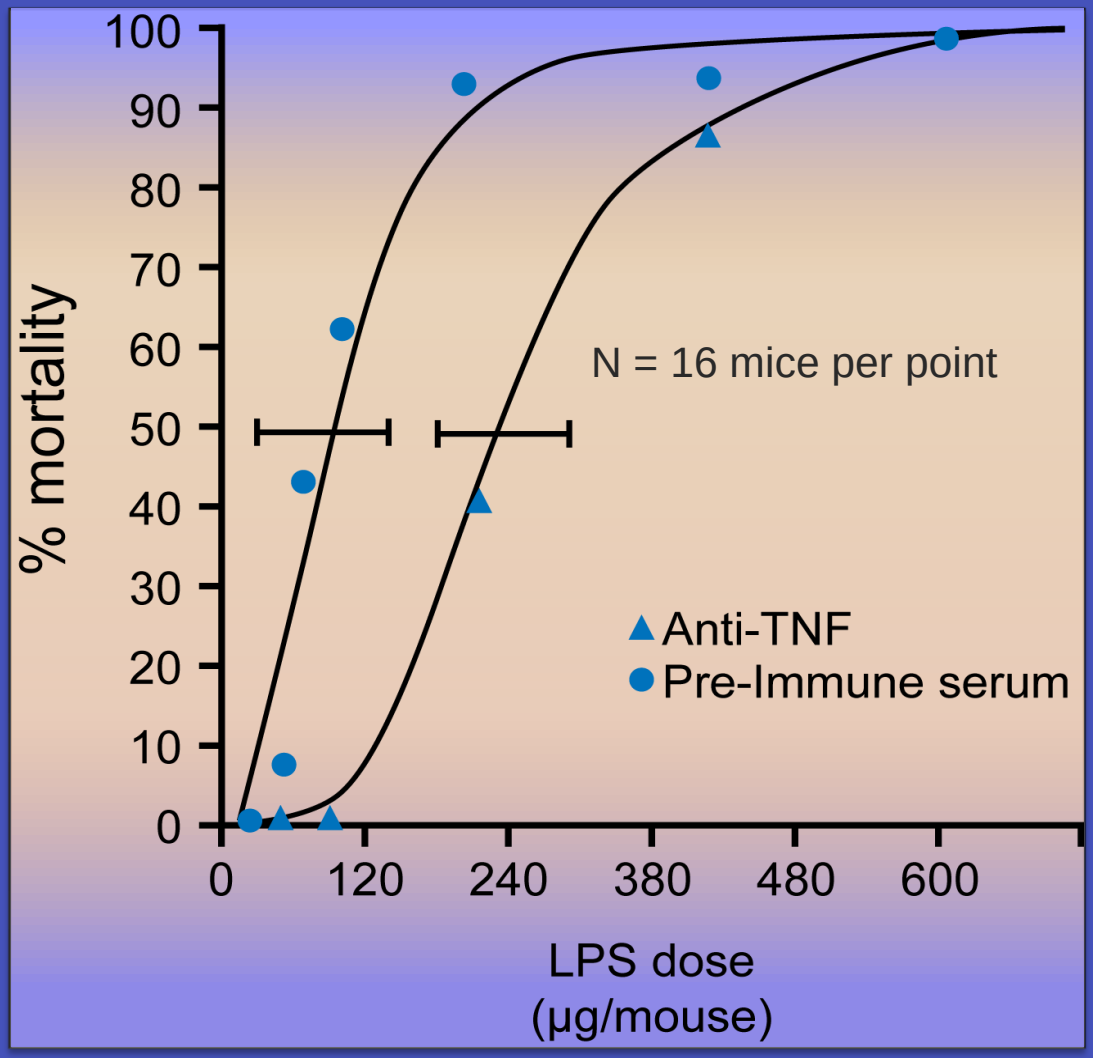
↑ Tumor necrosis and cytolysis

Purified TNF mimics LPS in its toxicity



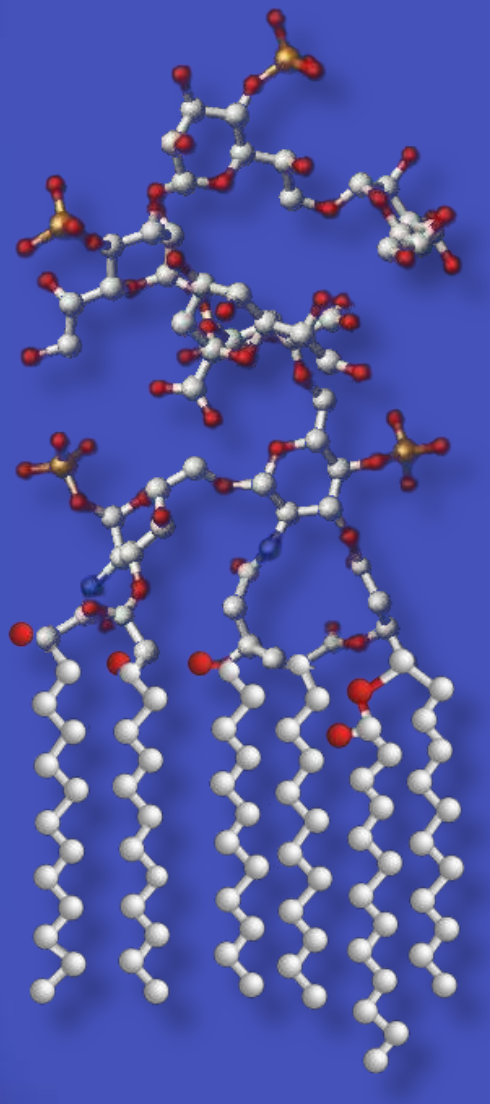
1984

The lethal effect of LPS is attenuated by passive immunization against TNF



1985

LPS



?

Macrophage

TNF

(And Other Cytokines)

Other Cells

NO

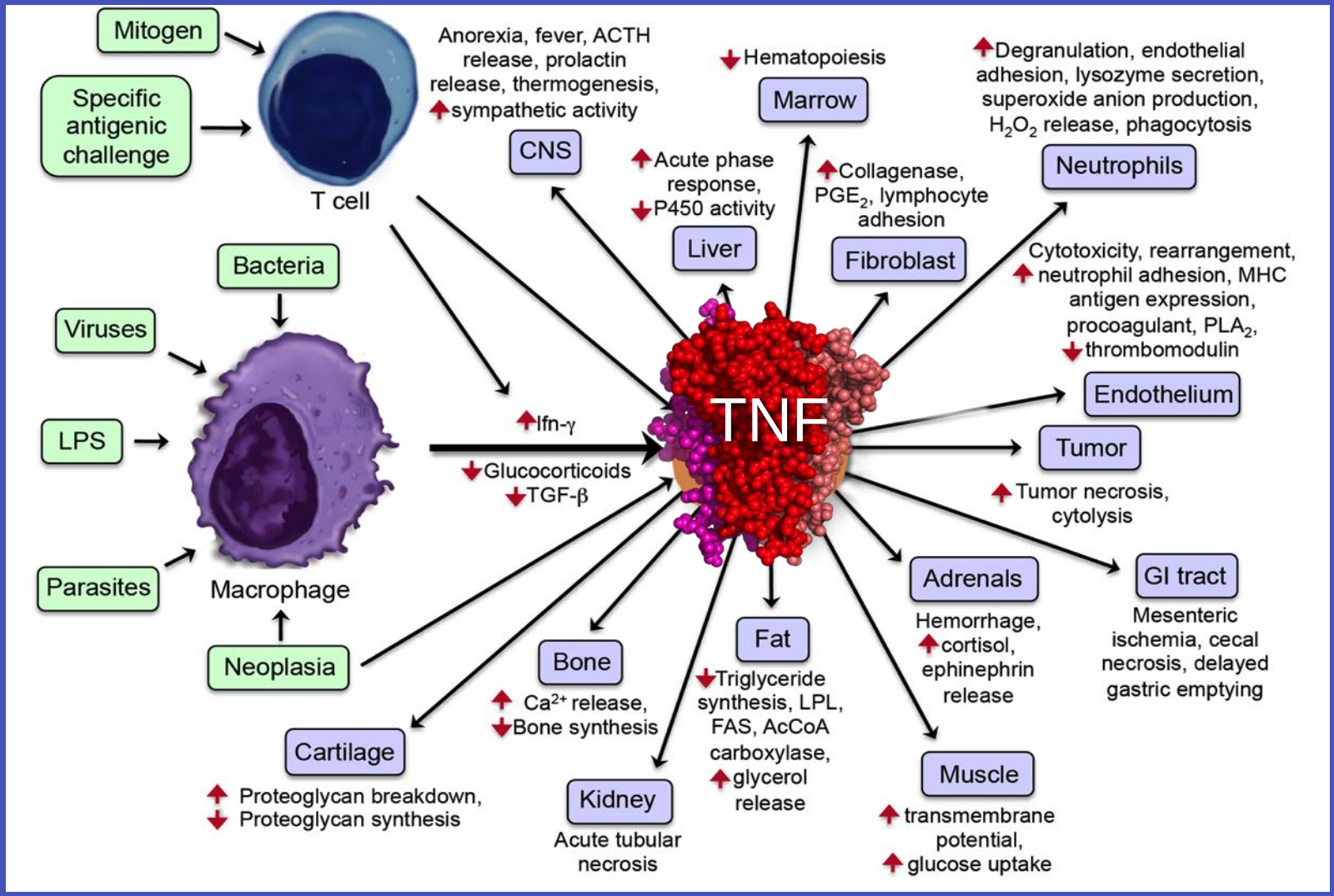
PAF

O_2^-

LT

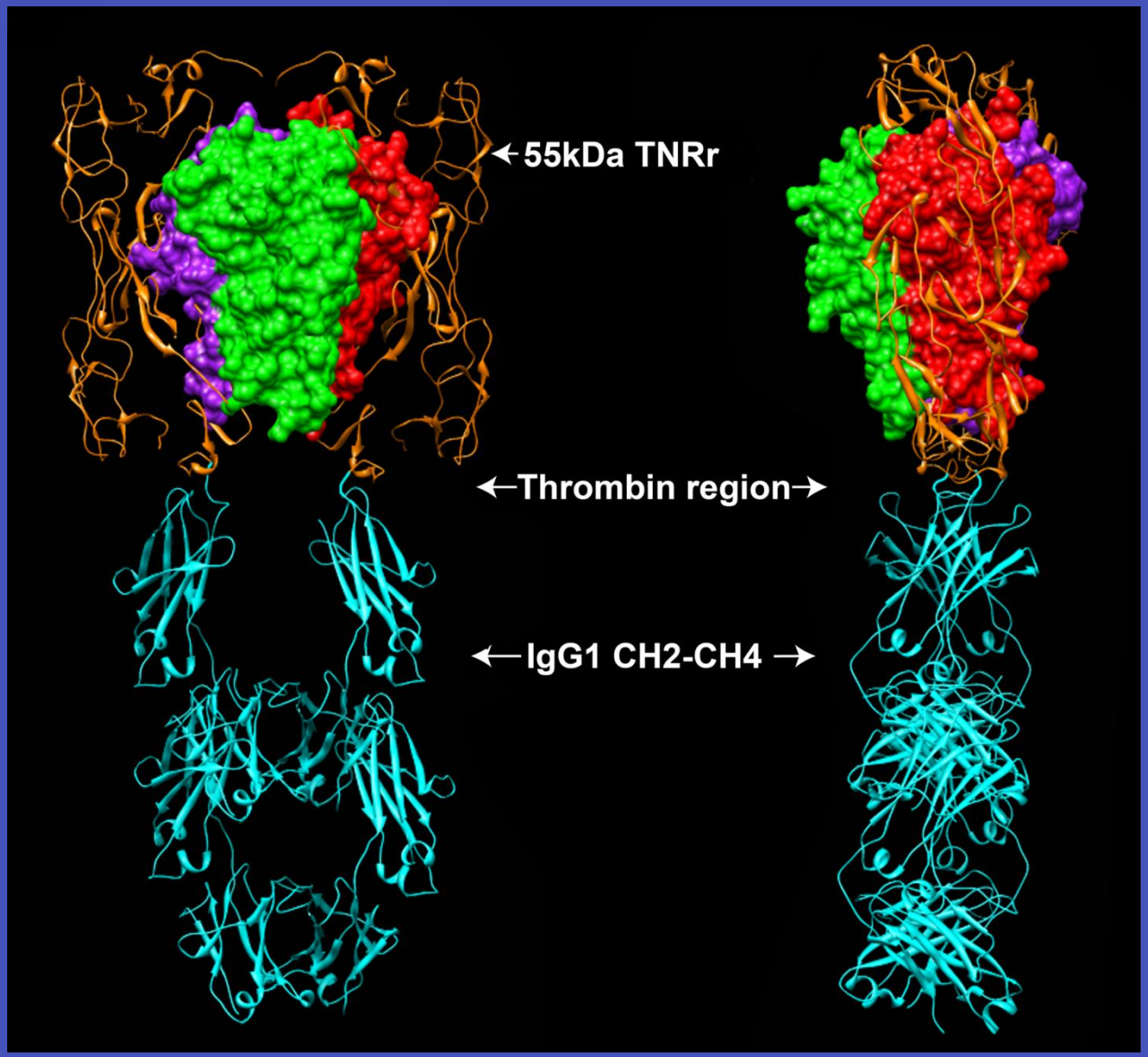
Kinins

1985


Mitogen
Specific antigenic challenge
T cell
Anorexia, fever, ACTH release, prolactin release, thermogenesis, ↑sympathetic activity
CNS
↓Hematopoiesis
Marrow
↑Degranulation, endothelial adhesion, lysozyme secretion, superoxide anion production, H_2O_2 release, phagocytosis
Neutrophils
↑Acute phase response, ↓P450 activity
Liver
↑Collagenase, PGE_2, lymphocyte adhesion
Fibroblast
Cytotoxicity, rearrangement, ↑neutrophil adhesion, MHC antigen expression, procoagulant, PLA_2, ↓thrombomodulin
Endothelium
Tumor
↑Tumor necrosis, cytolysis
Bacteria
Viruses
LPS
Parasites
Macrophage
Neoplasia
↑Ifn-γ
↓Glucocorticoids
↓TGF-β
TNF
Adrenals
Hemorrhage, ↑cortisol, ephinephrin release
GI tract
Mesenteric ischemia, cecal necrosis, delayed gastric emptying
Fat
↓Triglyceride synthesis, LPL, FAS, AcCoA carboxylase, ↑glycerol release
Bone
↑ Ca^{2+} release, ↓Bone synthesis
Cartilage
↑ Proteoglycan breakdown, ↓ Proteoglycan synthesis
Kidney
Acute tubular necrosis
Muscle
↑transmembrane potential, ↑glucose uptake

Looking at a model of a recombinant TNF inhibitor

1991



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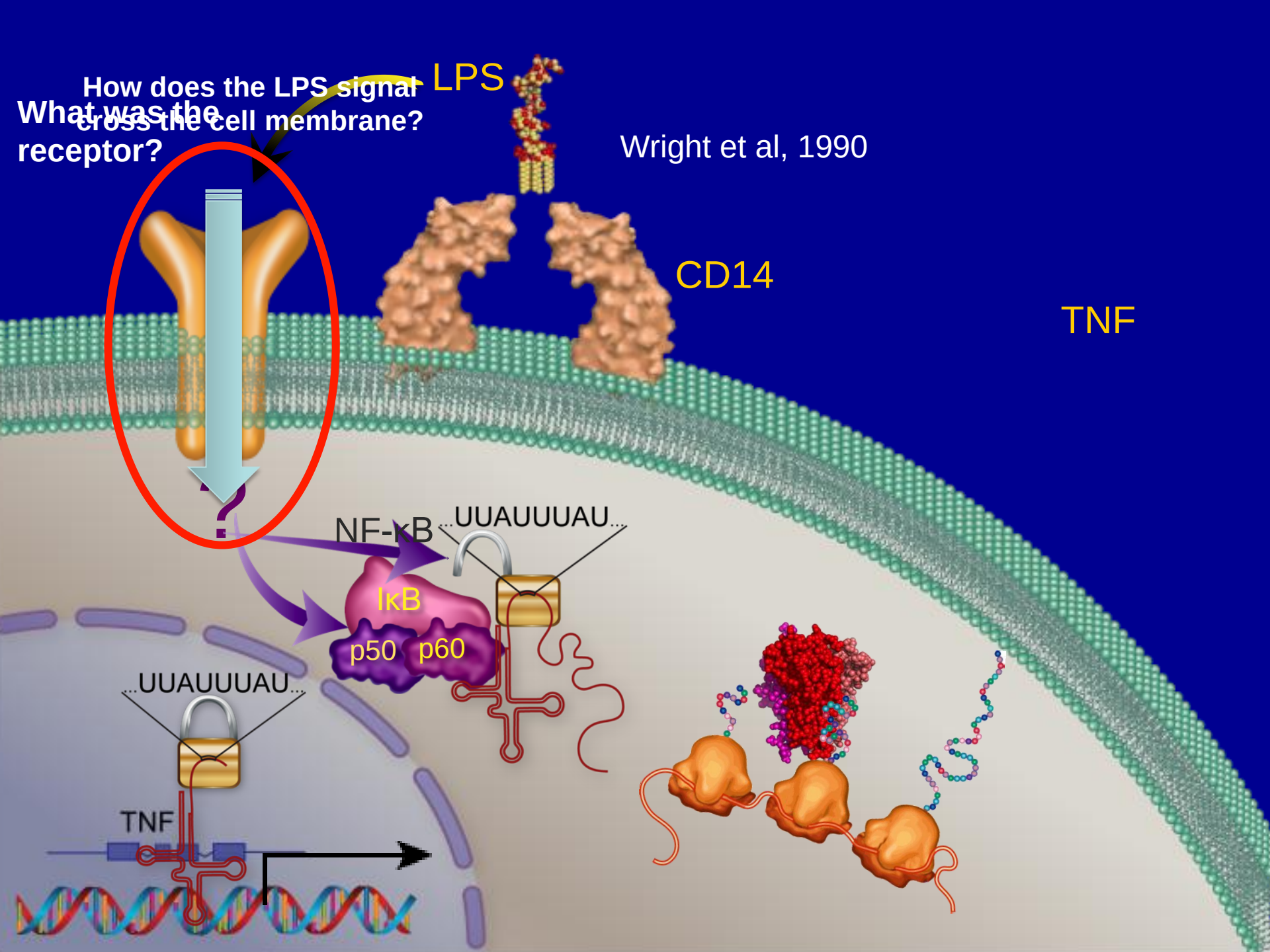
Peppel et al, *JEM:* 174(6):1483-9, 1991

All the while, I wondered about how LPS was perceived by cells and gradually came to see this as the most important question in the field of immunity and inflammation.

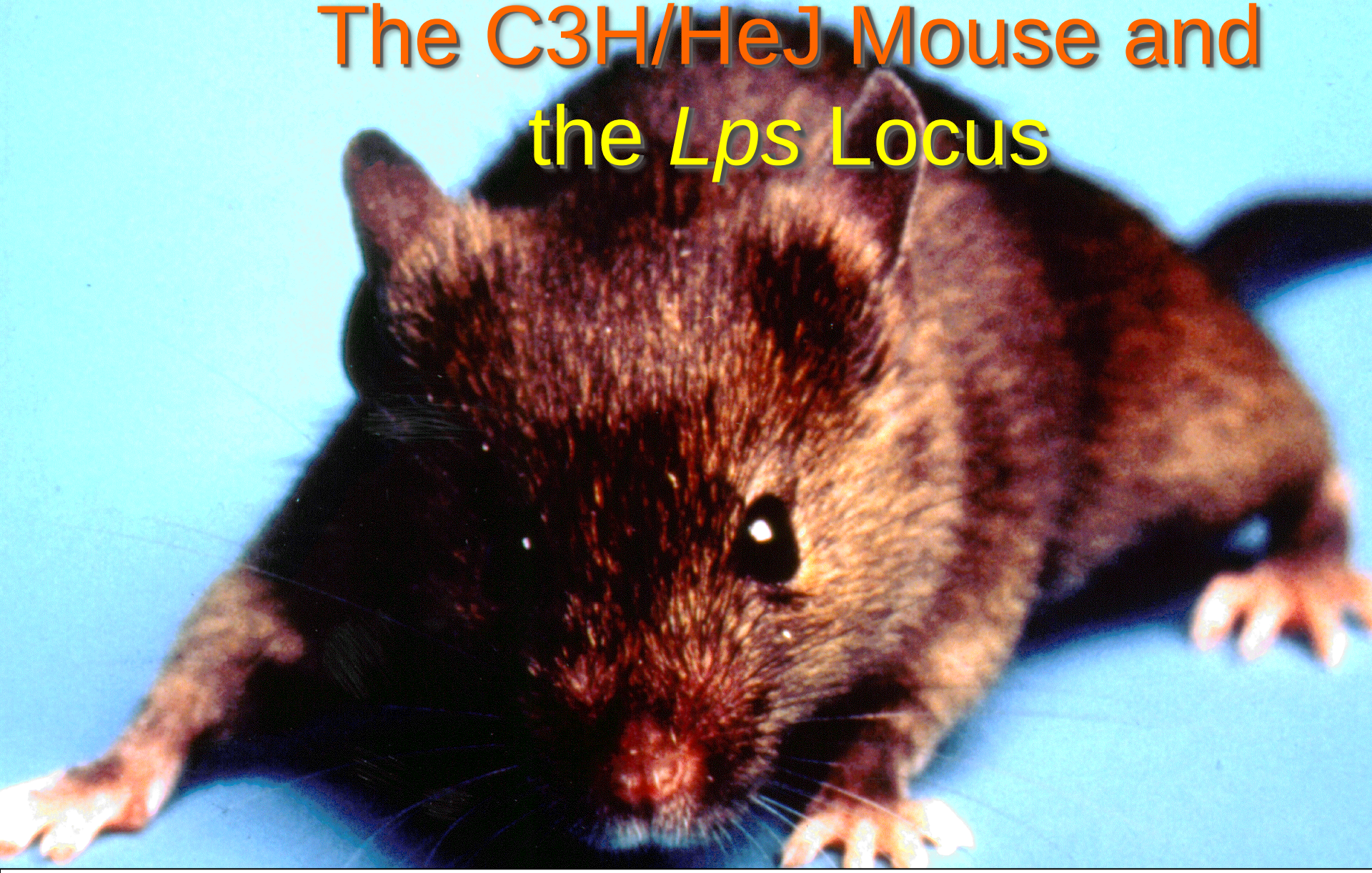
- We still did not know how innate immune recognition worked, and this seemed an excellent entry point into that question.
- To this day, we don't know what drives sterile inflammation. Here too, I thought we might find molecules of primary importance in this class of diseases (RA, ankylosing spondylitis, others).


How does the LPS signal cross the cell membrane?
What was the receptor?
LPS
Wright et al, 1990
CD14
TNF
NF-κB
UUAUUUAU
IκB
p50
p60
UUAUUUAU
TNF

We made several attempts to find the LPS receptor using immunological methods, protein chemistry, and expression cDNA cloning. All these approaches were unsuccessful.

The LPS receptor was ultimately found using a pure genetic approach, based on two unrelated substrains of mice that couldn't respond to LPS.

The C3H/HeJ Mouse and the *Lps* Locus



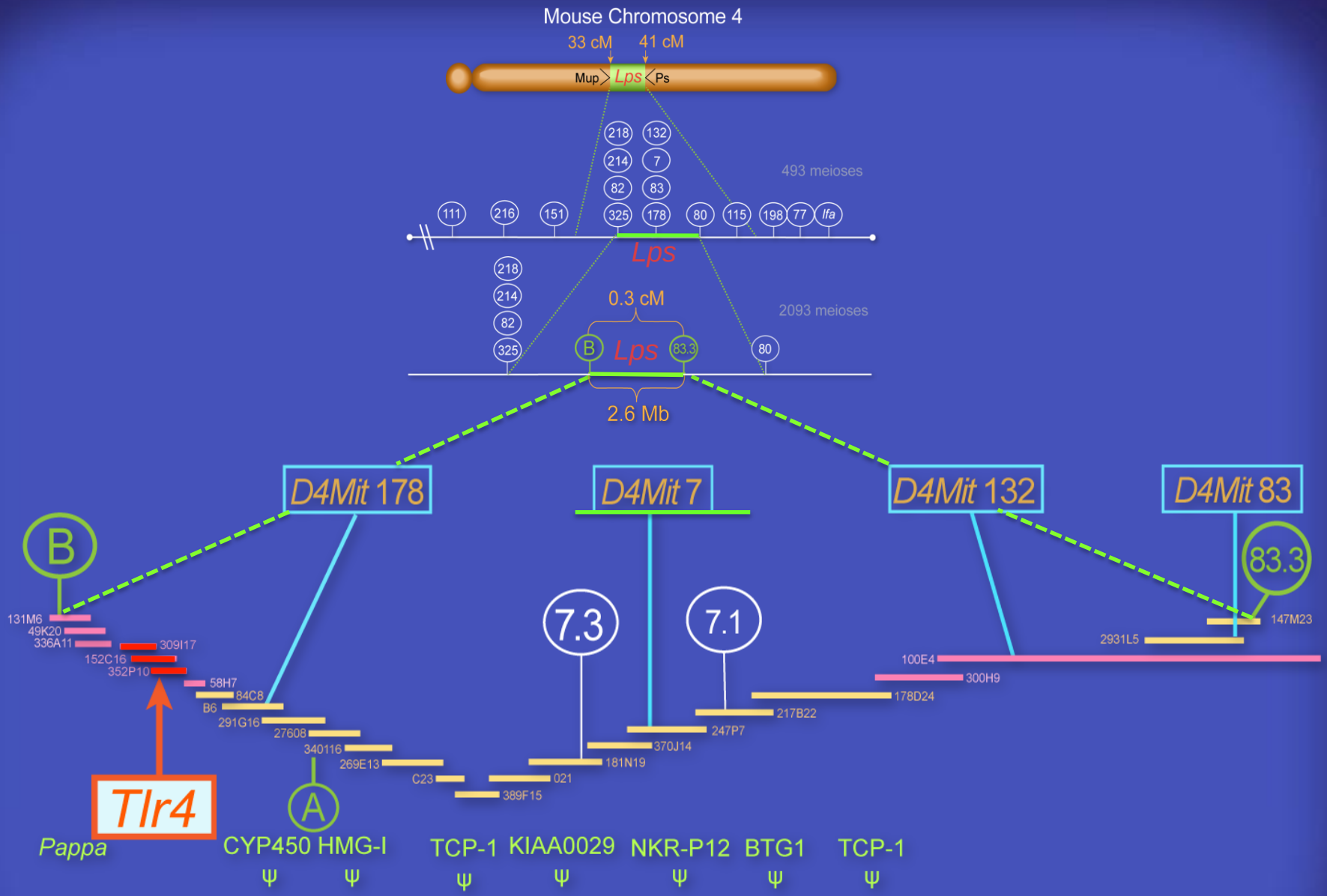
- Resistant to LPS (Heppner and Weiss, 1965), but highly susceptible to Salmonella (O'Brien, 1980)

The C57BL/10ScCr Mouse and the *Lps* Locus

- The same gene was affected in the LPS-refractory C57BL/10ScCr strain (Coutinho and Meo,1978).
- Located on Chr 4 (Watson and Riblet, 1978)

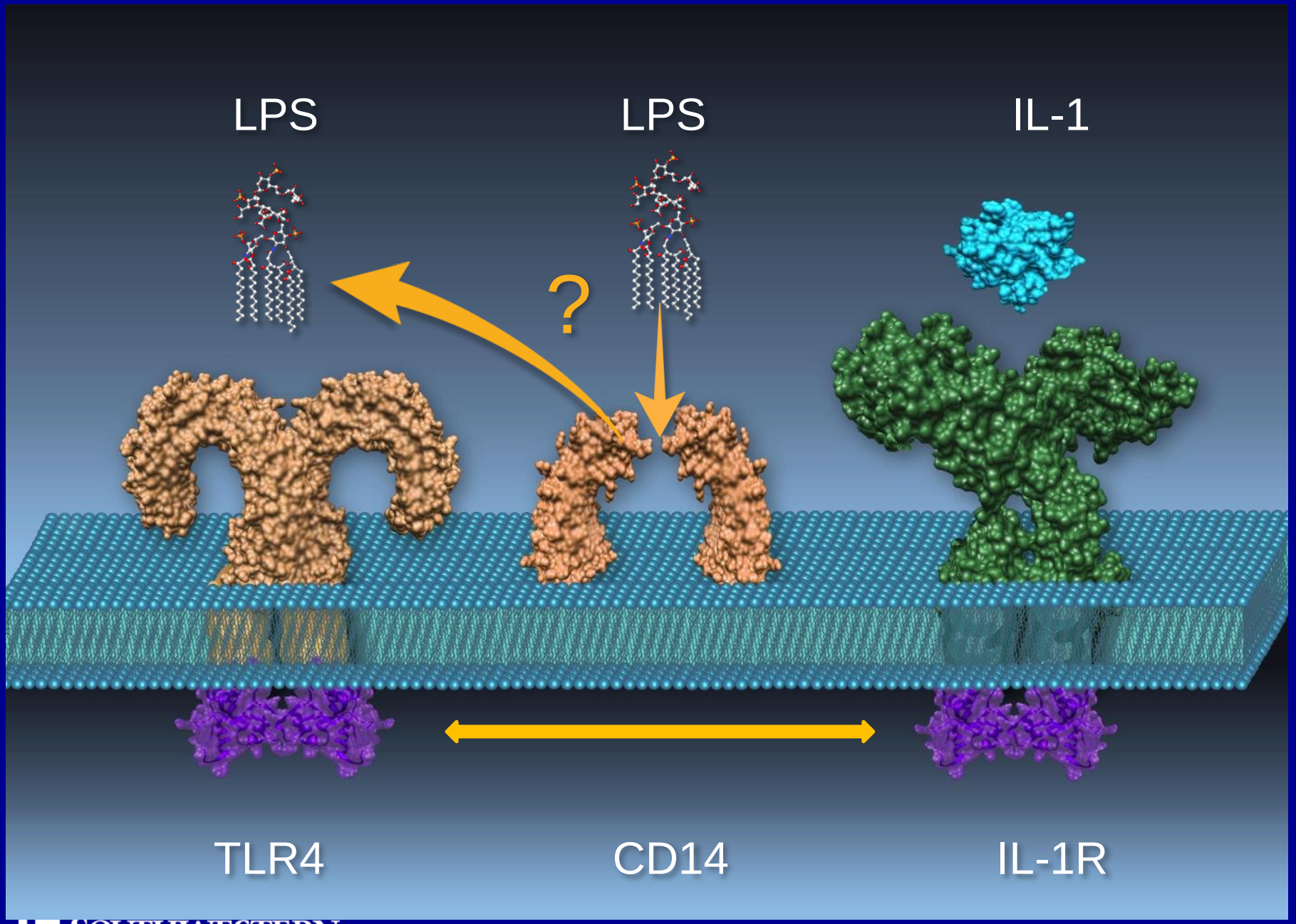
Beginning in 1993, we turned to positional cloning to try to find which gene was affected.

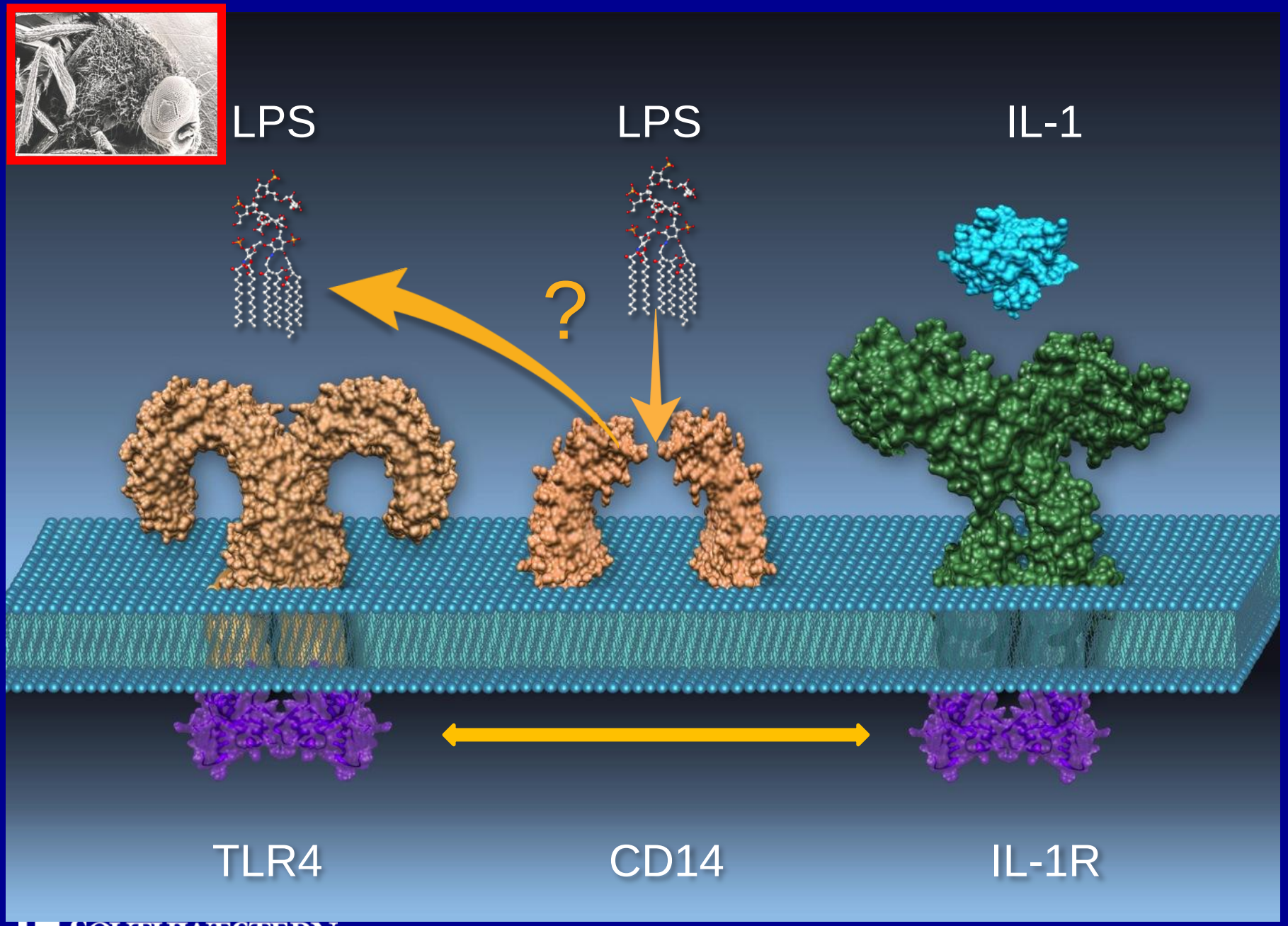
- Positional cloning = cloning by phenotype.
- One first establishes a “critical region,” delimited by markers in the genome, from which the phenotype emanates (genetic mapping)
- One then clones all the genomic DNA across the critical region, and identifies all the genes residing within it (physical mapping)
- Finally, one finds the causative mutation by comparing sequences of affected and control strains.



By summer of 1998, more than 90% of the critical region had been explored.

2.6 Mb

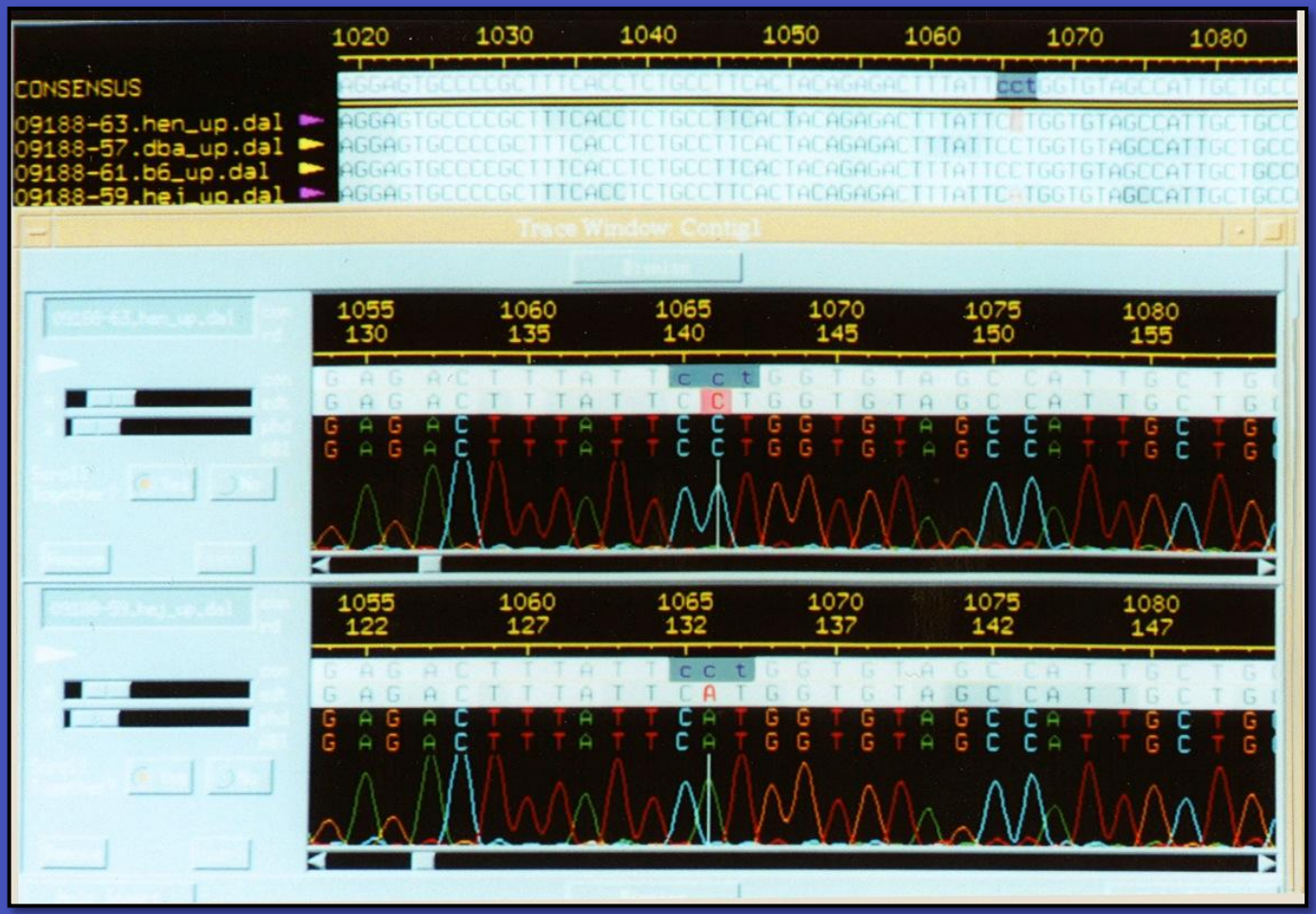

LPS
LPS
IL-1
?
TLR4
CD14
IL-1R


LPS
LPS
IL-1
?
TLR4
CD14
IL-1R

The mutation in *Tlr4* distinguishing C3H/HeJ from C3H/HeN mice

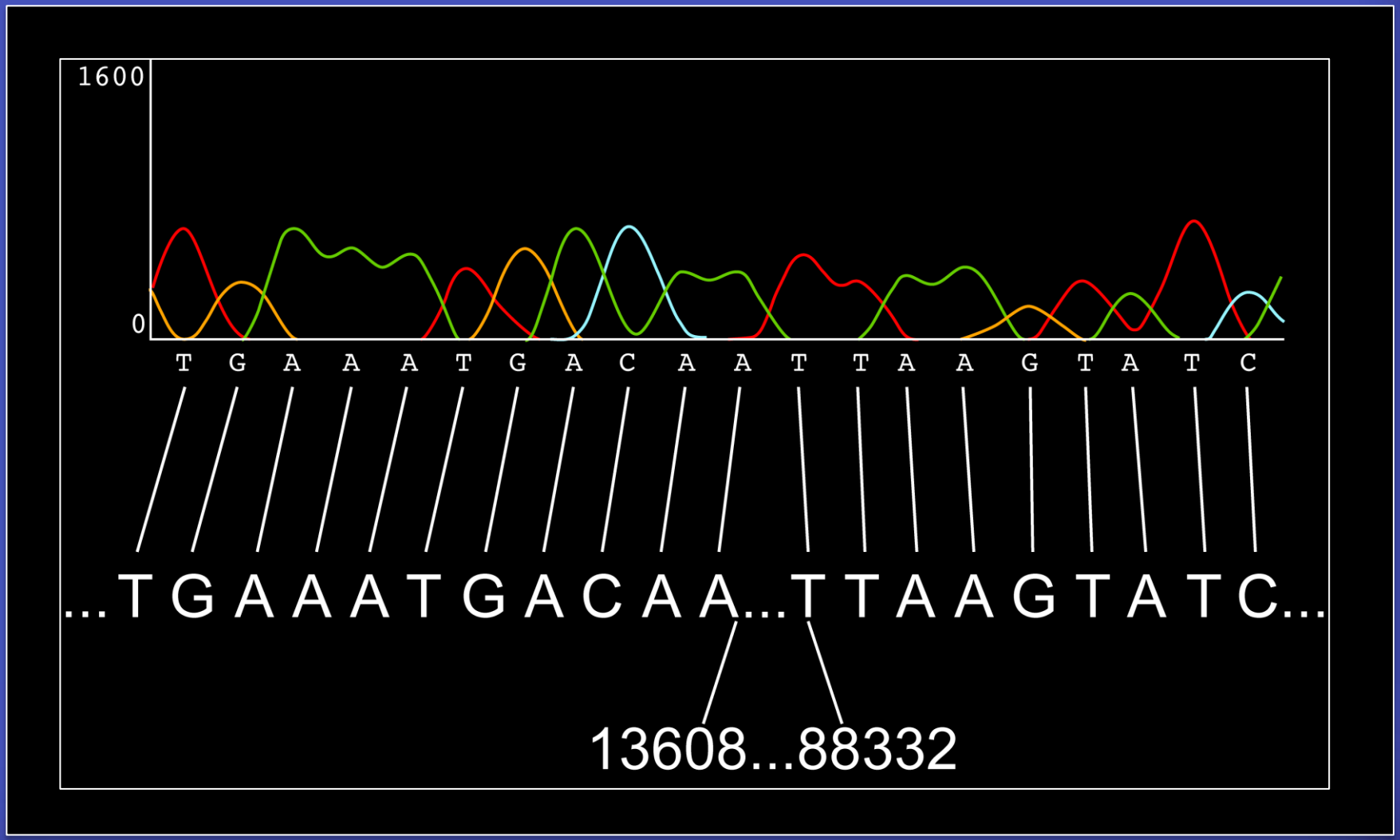
C3H/HeN

C3H/HeJ

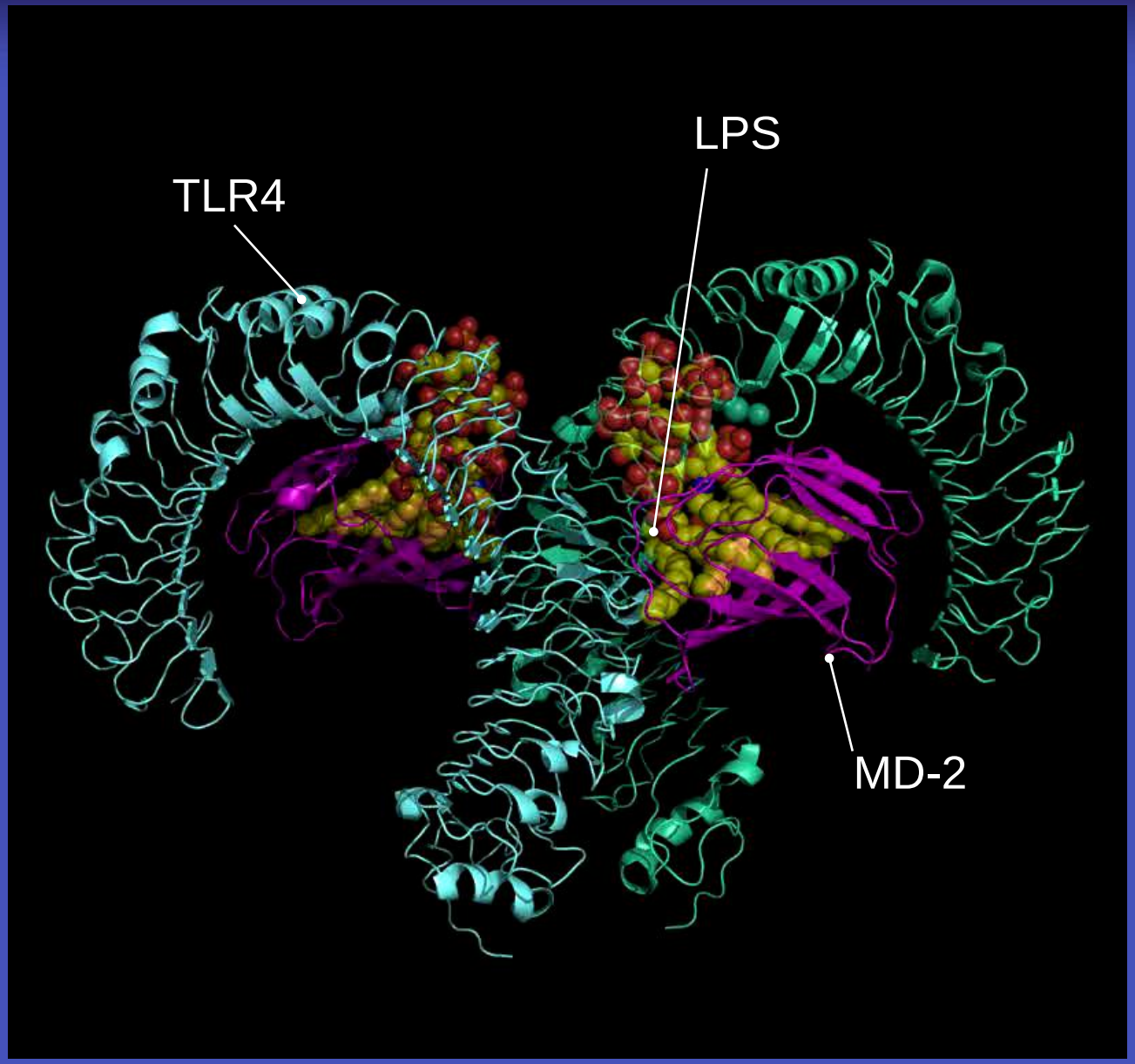


1998

Deletion of 74 kb in the C57BL/10ScCr mouse

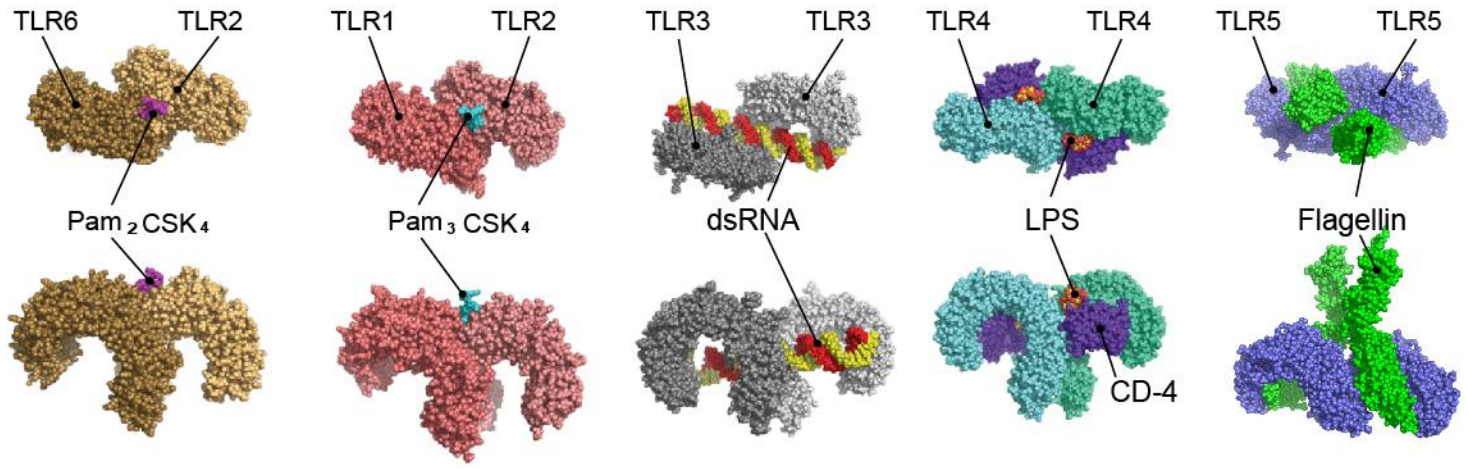


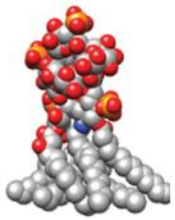
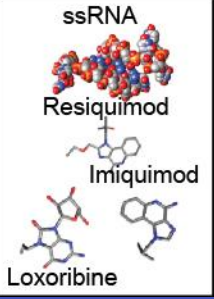
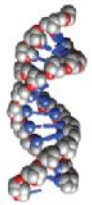
1998



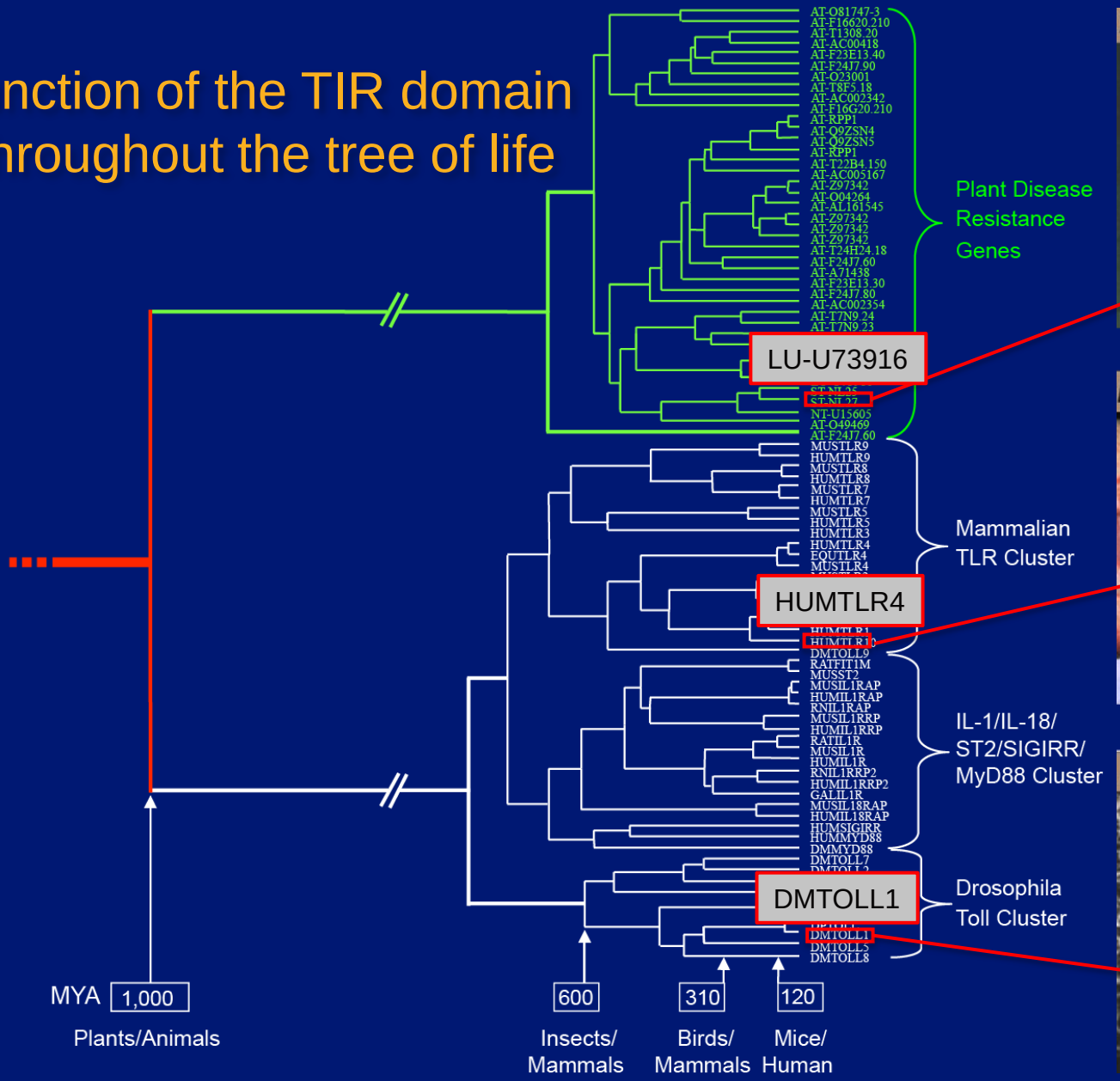
Crystal structure of the TLR4/MD-2/Lipid-A complex was published by Jie-Oh Lee (Park et al) in 2009

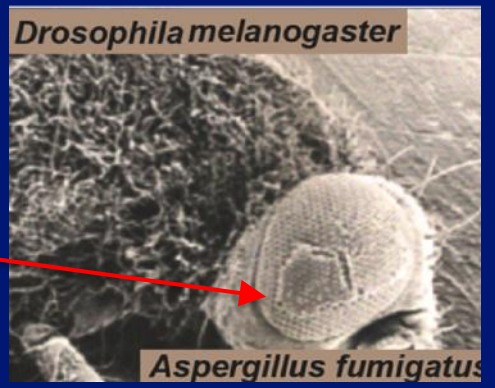
Now, the mode of binding of several ligands to TLRs is understood



Receptor	TLR 1/2 or 2/6	TLR3	TLR4	TLR5	TLR7	TLR9
Ligands	Lipoproteins	Poly I:C, dsRNA	LPS	Flagellin	ssRNA	CpG-DNA
Source	Gram pos bacteria, fungi	Viruses	Gram neg bacteria	Bacterial flagellum	Viruses	Bacteria
Examples						

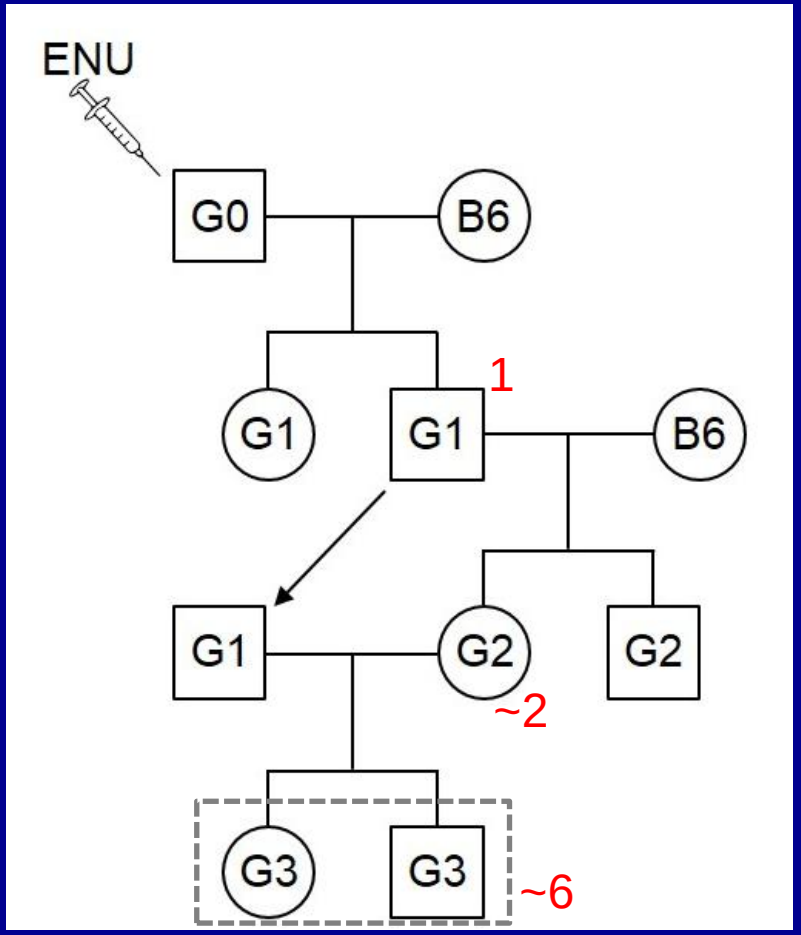
Function of the TIR domain throughout the tree of life

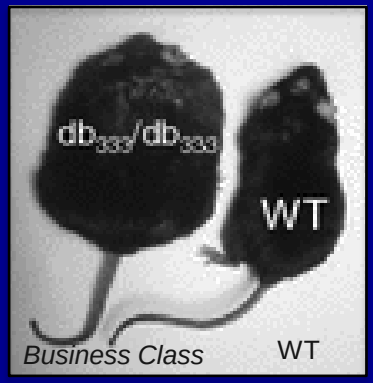




- The next set of questions had to do with the signaling pathway utilized by TLR4.
- There were no other spontaneous mutations in mice that could inform us of how LPS signaling worked.
- We decided to create new phenotypes using a mutagen (work that began in 2000).
- Many other aspects of immunity interested us as well, and these too were placed under surveillance in screens for phenovariance.

Making new phenotypes in mice to understand TLR signaling: mutagenesis with ENU





An average of 60-70 coding changes are represented in every G1 progenitor.

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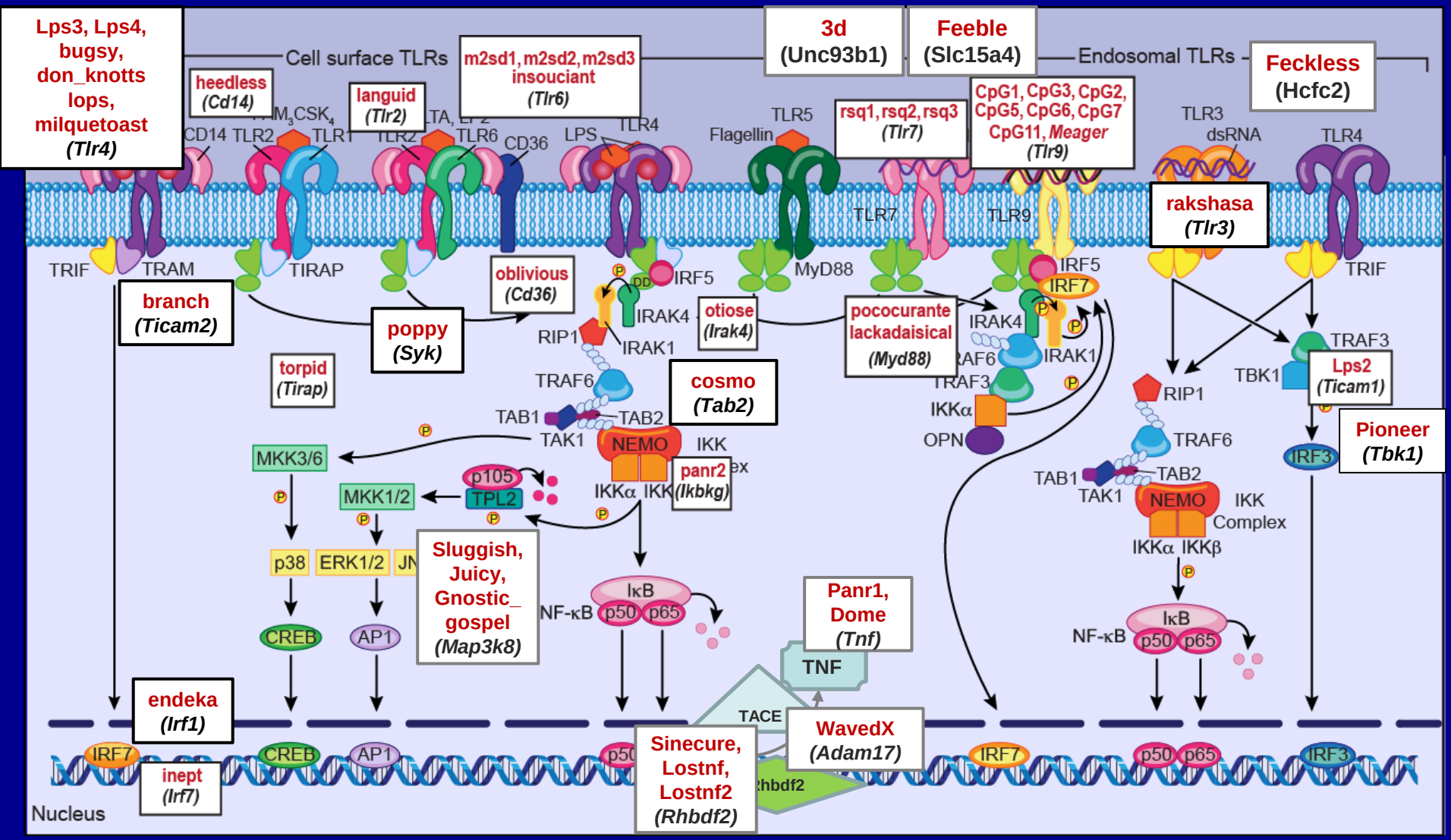
50 mutations in 26 genes were detected because of TLR signaling defects



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Mutation finding was accelerated by technical innovations over this period, but remained much too slow, and was a blind process.

By 2011, it was clear that the rate limiting step in mutation finding was genetic mapping.

Stock establishment, outcrossing, backcrossing, genotyping, and phenotyping to establish location took much too long.

Many more phenotypes were declared than could be found.

A new approach was needed.

We wished for a magical tool that would permit instant resolution of phenotypes to a single base pair change.

We wished for a magical approach that would permit instant resolution of phenotypes to a single [illegible]

- Gene symbol: *Sox10*
- Mutation: 15:79,163,324 T -> G
- Protein change: N130K of 466 residues
- Motif: DNA-Binding High Mobility Group
- Human orthologue: SOX10

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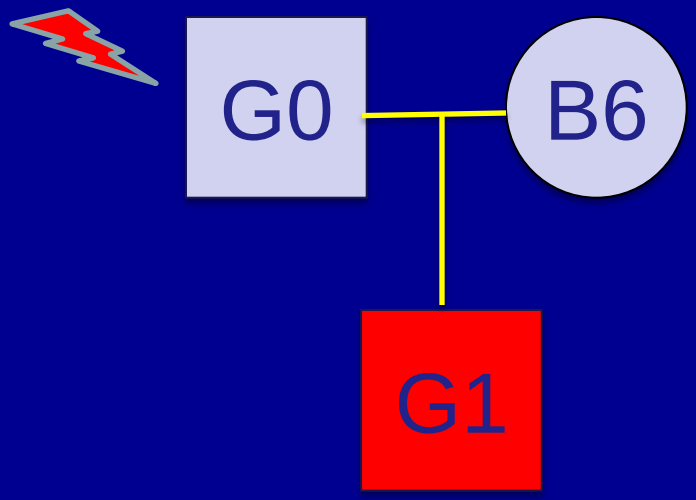
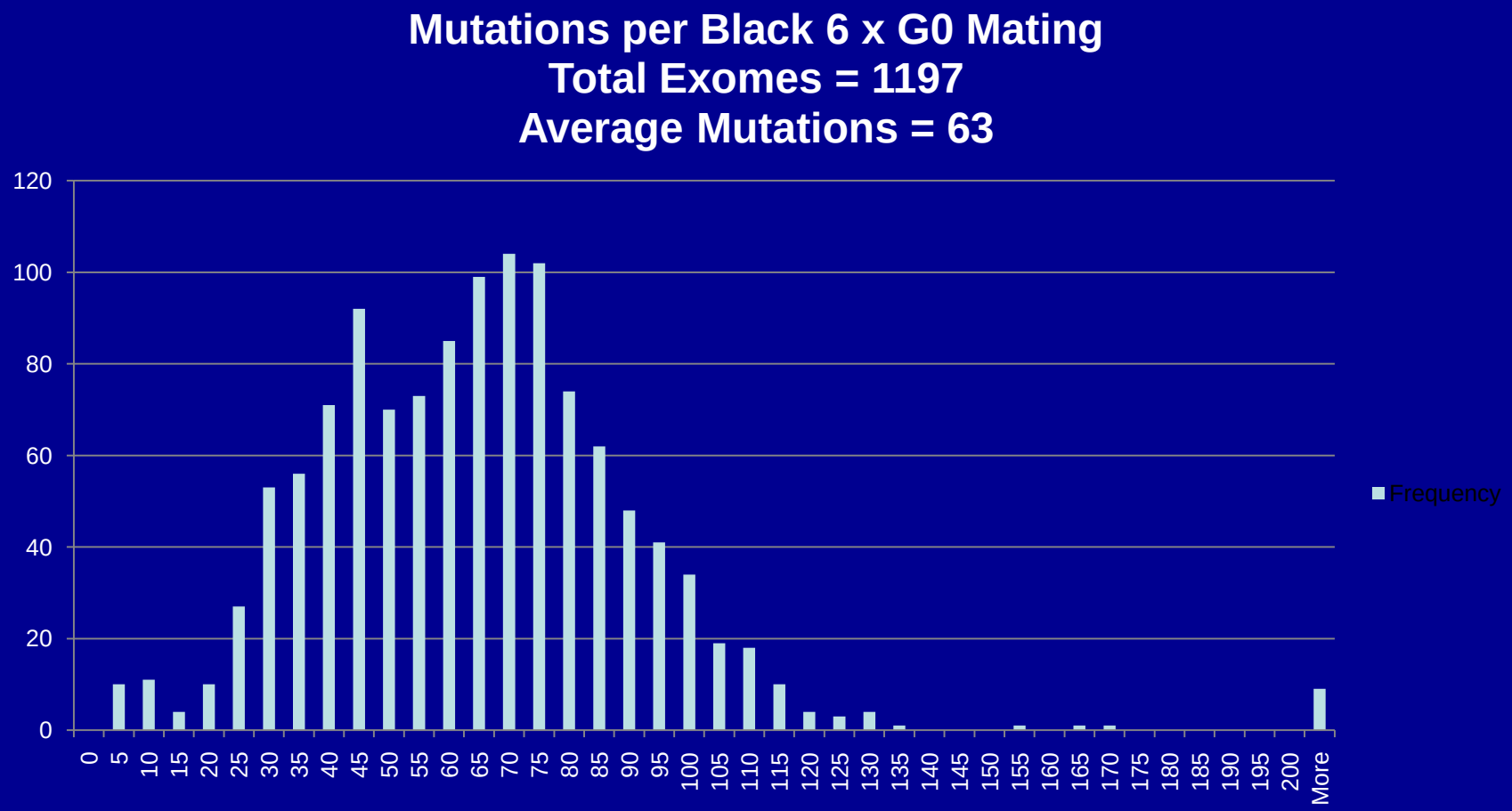
How it is done…

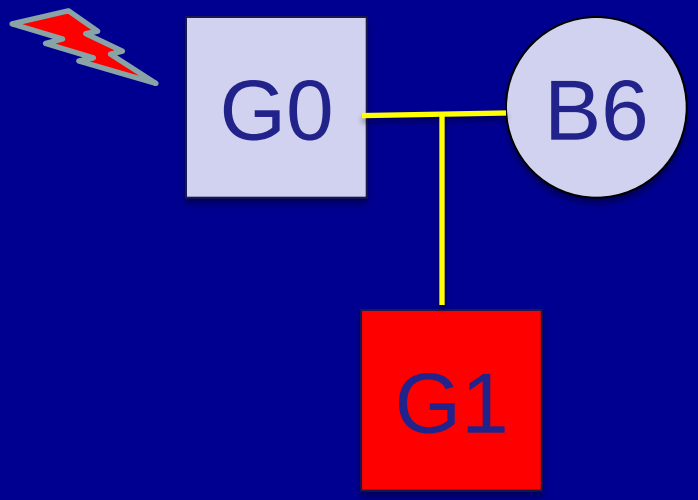


First we make the G1 mice…

Then we whole-exome sequence all G1 mice to find all mutations they might transmit to the G2 an G3 mice.

B6 x G0 mutations

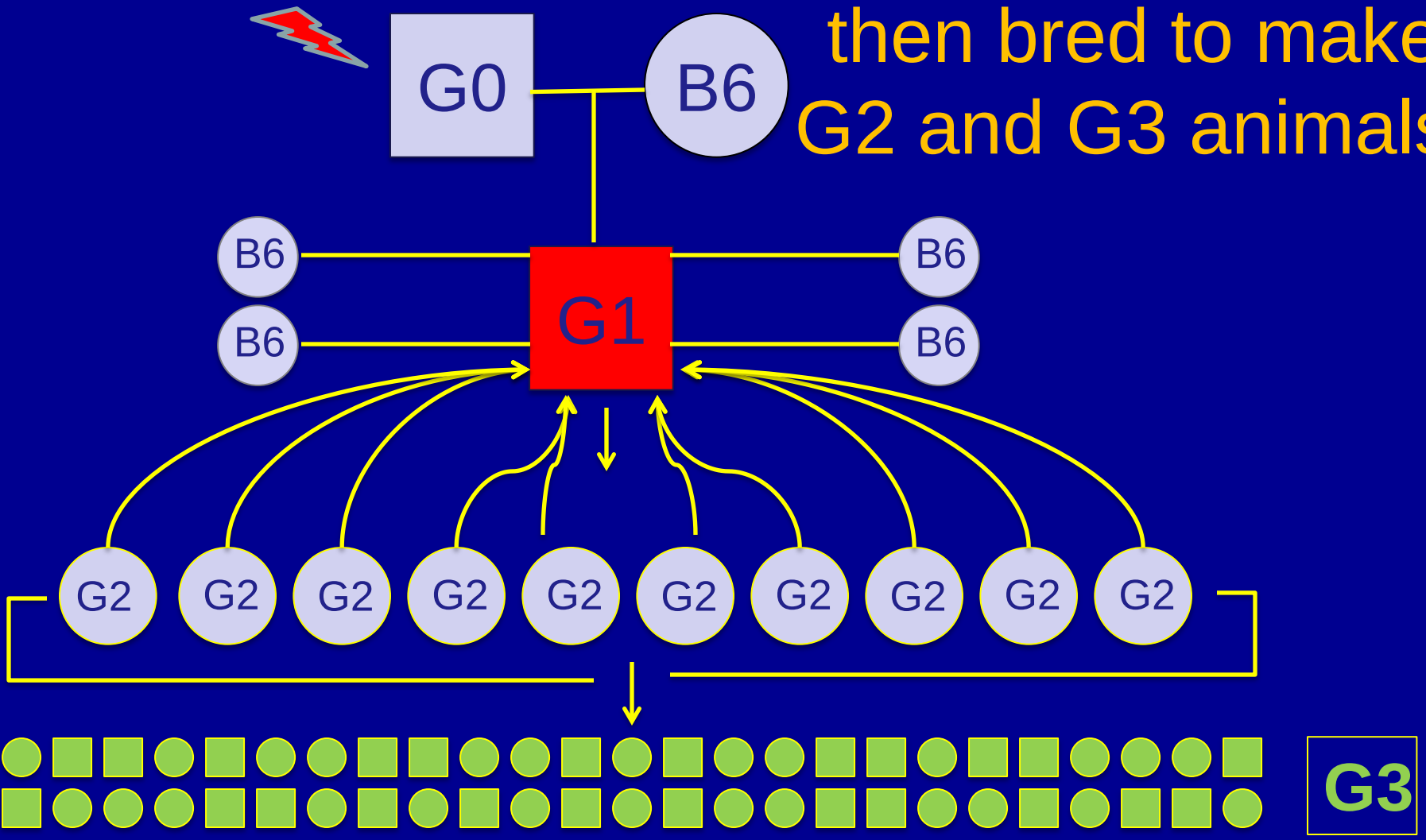




First we make the G1 mice…

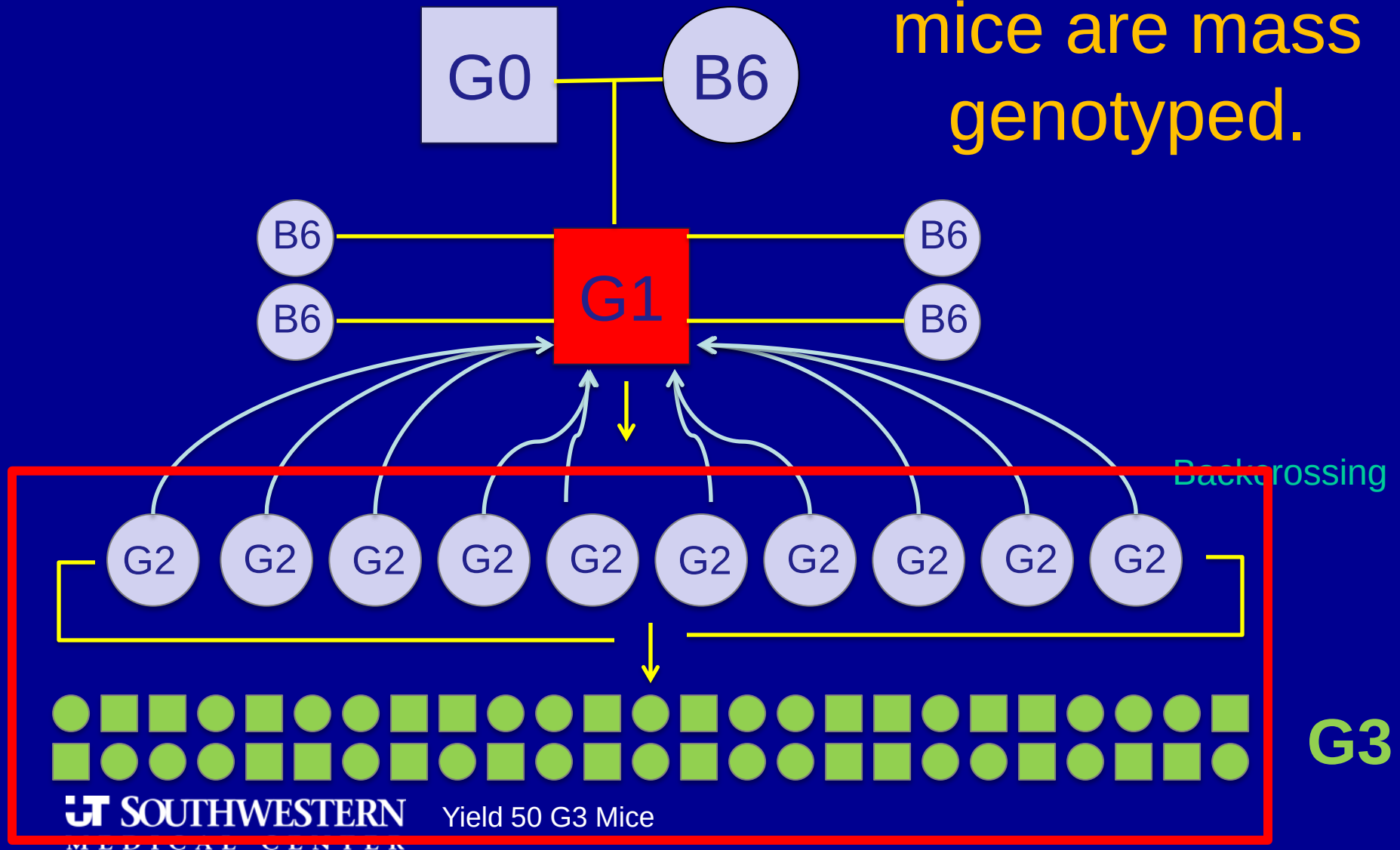
Then we whole-exome sequence all G1 mice to find all mutations they might transmit to the G2 an G3 mice. If >30 mutations are present, we move forward to order an Ampliseq panel.

The G1 mice are then bred to make G2 and G3 animals.



Yield 50 G3 Mice

The G0', G2 and G3 mice are mass genotyped.



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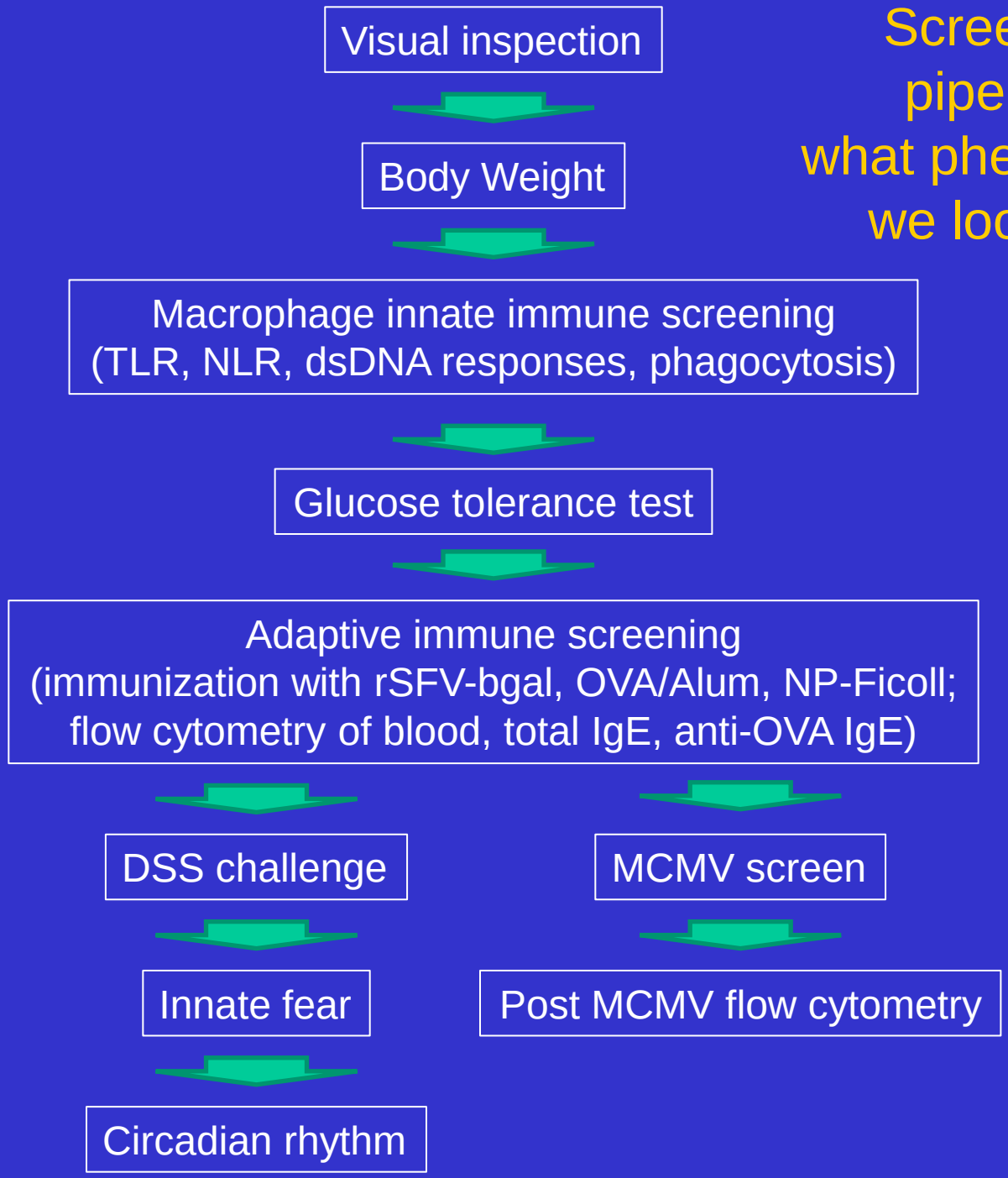
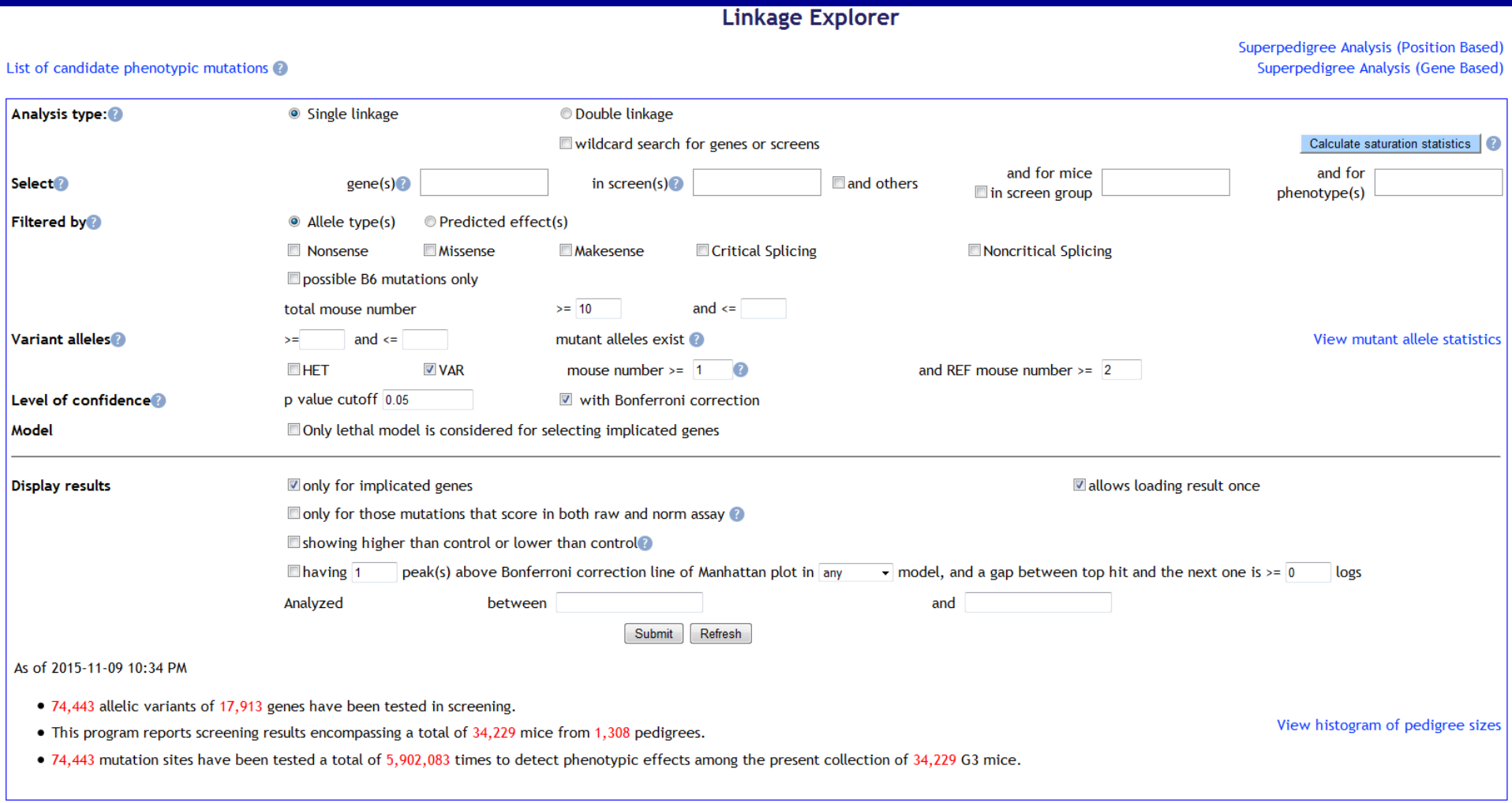
Screening pipeline: what phenotypes we look for.



Summary statistics as of Nov. 9, 2015…

- 74,443 allelic variants of 17,913 genes have been tested in screening. Total gene number = 24,981 (72% affected)
- These mutations resided within a total of 34,229 G3 mice from 1,308 pedigrees.
- Approximately 19.7% of the genome has been mutated “to phenovariance” and tested in the homozygous state 3x or more in one or more screens.
- In adaptive immune screens alone, causative mutations in >70 genes known to be needed for immune development or function have been found, along with mutations in hundreds of genes that were previously unknown.
- A large fraction of our genome is needed for immune defense.

Linkage explorer: a program for the retrieval of relevant linkage data from large datasets



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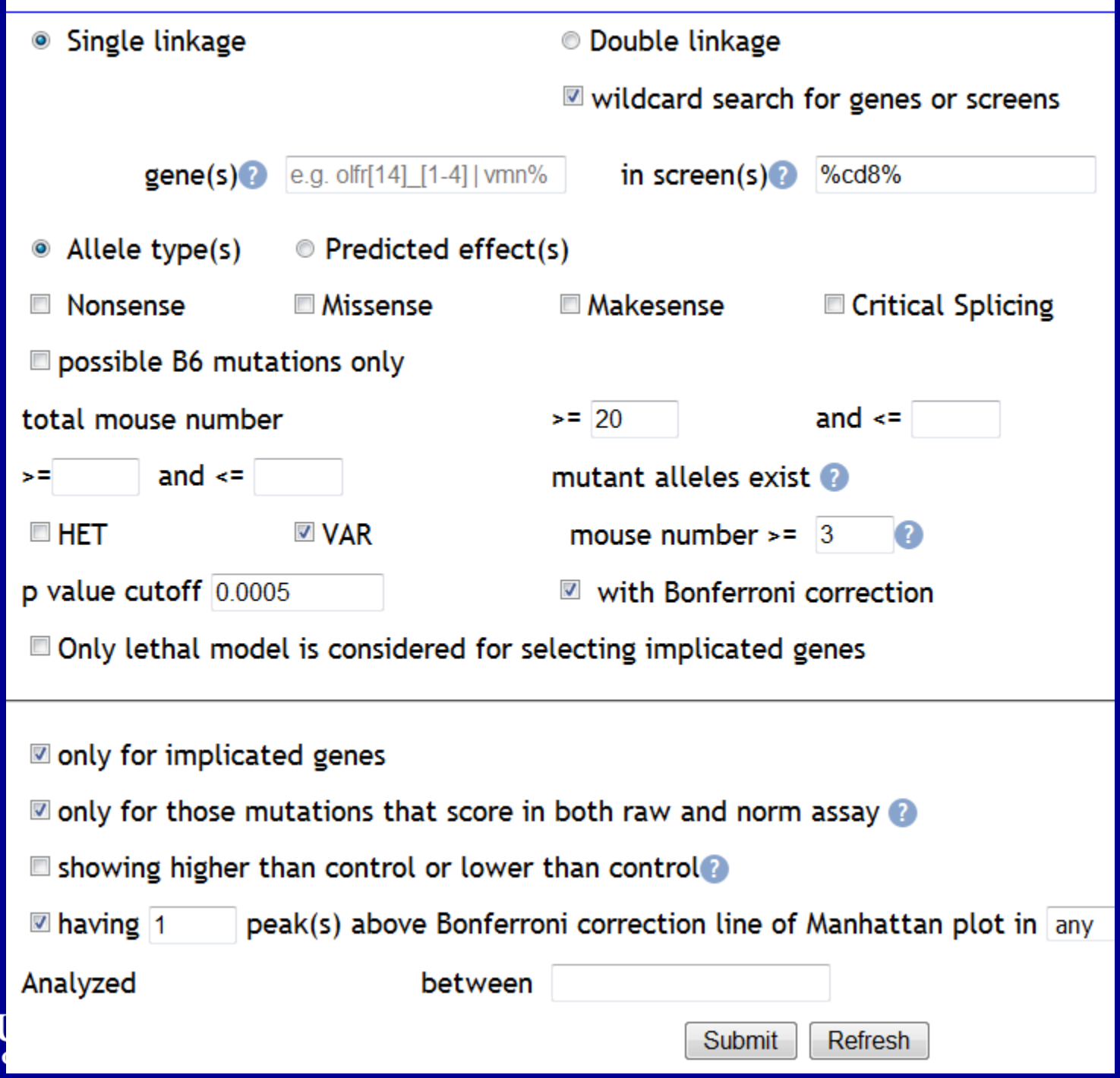

Single linkage
Double linkage
wildcard search for genes or screens
gene(s)
e.g. olfr[14]_[1-4] | vmn%
in screen(s)
%cd8%
Allele type(s)
Predicted effect(s)
Nonsense
Missense
Makesense
Critical Splicing
possible B6 mutations only
total mouse number
>= 20
and <=
>=
and <=
mutant alleles exist
HET
VAR
mouse number >= 3
p value cutoff 0.0005
with Bonferroni correction
Only lethal model is considered for selecting implicated genes
only for implicated genes
only for those mutations that score in both raw and norm assay
showing higher than control or lower than control
having 1 peak(s) above Bonferroni correction line of Manhattan plot in any
Analyzed
between
Submit
Refresh

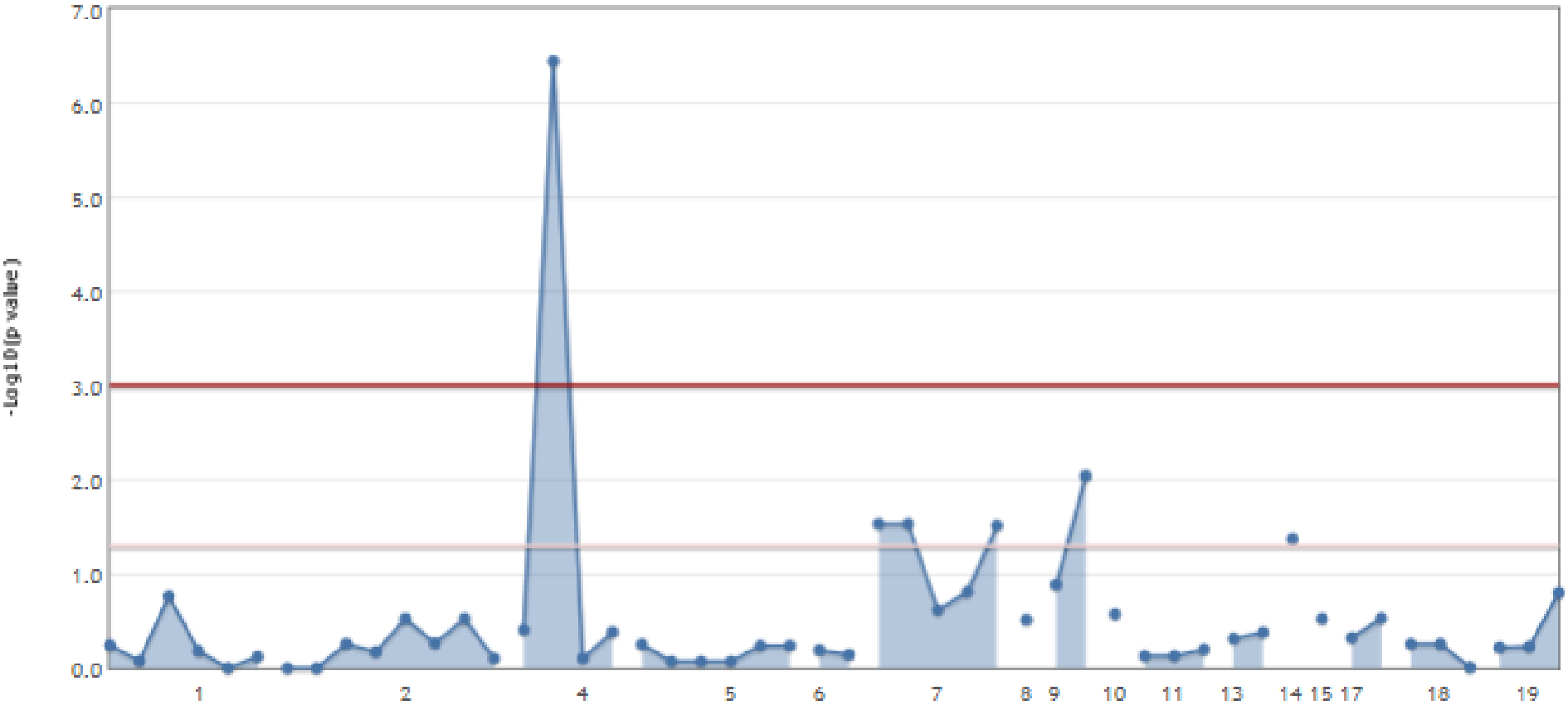
The search returns a list of 104 variant alleles of 100 implicated genes, derived from 71 pedigrees.

31		Snrnp40 {4/20}	sp	4_130378043	splice acceptor site		probably null	R0443	FACS CD8+ T cells
32		Snrnp40 {4/20}	sp	4_130378043	splice acceptor site		probably null	R0443	FACS CD8+ T cells
33		Snrnp40 {4/20}	sp	4_130378043	splice acceptor site		probably null	R0443	FACS CD44+ CD8 MFI
34		Snrnp40 {4/20}	sp	4_130378043	splice acceptor site		probably null	R0443	FACS CD44+ CD8 MFI
35		Snrnp40 {4/20}	sp	4_130378043	splice acceptor site		probably null	R0443	FACS CD44+ CD8 MFI
36		Snrnp40 {4/20}	sp	4_130378043	splice acceptor site		probably null	R0443	FACS CD44+ CD8 MFI
37		Themis {6/6}	sp	10_28782011	missense	1.000	probably damaging	R0445	FACS CD4:CD8
38		Themis {6/6}	sp	10_28782011	missense	1.000	probably damaging	R0445	FACS CD4:CD8
39		Themis {6/6}	sp	10_28782011	missense	1.000	probably damaging	R0445	FACS CD4:CD8
40		Themis {6/6}	sp	10_28782011	missense	1.000	probably damaging	R0445	FACS CD8+ T cells in CD3+ T cells
41		Themis {6/6}	sp	10_28782011	missense	1.000	probably damaging	R0445	FACS CD8+ T cells in CD3+ T cells
42		Themis {6/6}	sp	10_28782011	missense	1.000	probably damaging	R0445	FACS CD8+ T cells in CD3+ T cells
43		Themis {6/6}	sp	10_28782011	missense	1.000	probably damaging	R0445	FACS CD8+ T cells in CD3+ T cells
44		Themis {6/6}	sp	10_28782011	missense	1.000	probably damaging	R0445	FACS CD8+ T cells in CD3+ T cells
45		Themis {6/6}	sp	10_28782011	missense	1.000	probably damaging	R0445	FACS CD8+ T cells in CD3+ T cells
46		Themis {6/6}	sp	10_28782011	missense	1.000	probably damaging	R0445	FACS CD8+ T cells in CD3+ T cells
47		Themis {6/6}	sp	10_28782011	missense	1.000	probably damaging	R0445	FACS CD8+ T cells in CD3+ T cells

Mht plot: recessive model (raw data wG2)

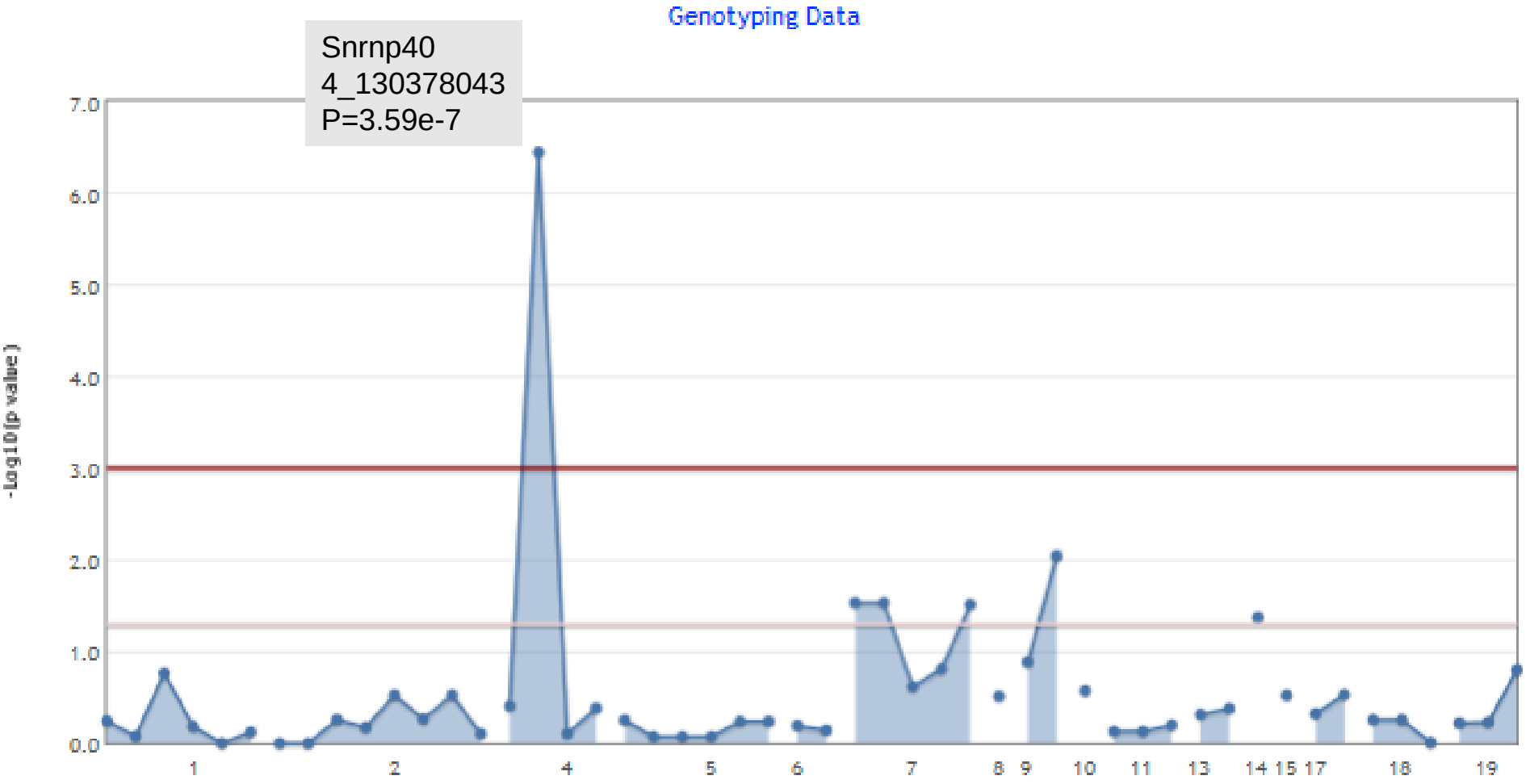
Single pedigree R0443 tested in FACS CD8+ T cells

Genotyping Data



Mht plot: recessive model (raw data wG2)

Single pedigree R0443 tested in FACS CD8+ T cells



ID	39263
Institutional Source	Beutler Lab
Gene Symbol	*Snrnp40*
Gene Name	small nuclear ribonucleoprotein 40 (U5)
Synonyms	Wdr57
Accession Numbers	
Run Code	HSQ01007
Stock #	R0443 (G1)
Quality Score	225
Status	Validated (trace)
Chromosome	4
Chromosomal Location	130360132-130390026 bp(+) (GRCm38)
Type of Mutation	splice acceptor site (3 bp from exon)
DNA Base Change (assembly)	C to G at 130378043 bp
Zygosity	Heterozygous
Amino Acid Change	
Ref Sequence	ENSEMBL: ENSMUSP00000101616 (fasta)
Gene Model	predicted sequence gene model

SMART Domains (Ref Sequence)

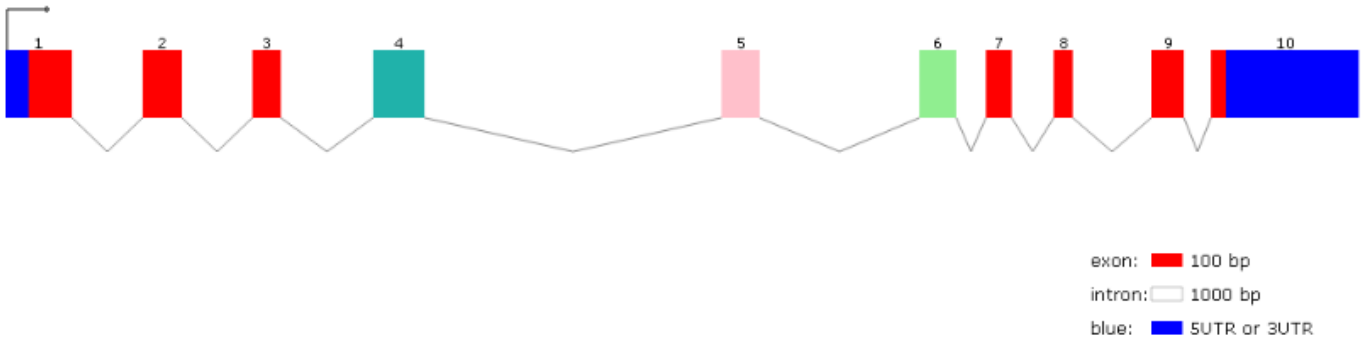
Domain	Start	End	E-Value	Type
low complexity region	24	45	N/A	INTRINSIC
WD40	56	95	1.64e-9	SMART
WD40	99	138	1.83e-7	SMART
WD40	141	181	8.68e-9	SMART
WD40	184	222	3.81e-5	SMART
WD40	225	264	3.24e-8	SMART
WD40	271	314	5.1e-6	SMART
WD40	317	356	2.84e-4	SMART

SMART Domains (Predicted Sequence)

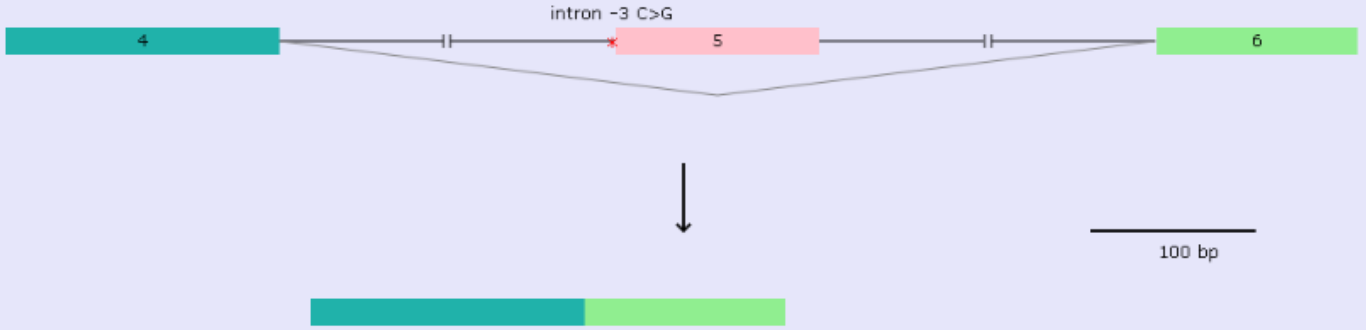
Domain	Start	End	E-Value	Type
low complexity region	24	45	N/A	INTRINSIC
WD40	56	95	9.2e-12	SMART
WD40	99	138	9.6e-10	SMART
WD40	141	181	3.4e-11	SMART
WD40	184	223	1.7e-10	SMART

Gene model

A. Gene View: Gene Symbol = Snrnp40; Transcript_ID = ENSMUST00000105994; Transcript_Name = Snrnp40-001



B. Mutation View: HSQ01007, R0443 at chr4_130378043 and Predicted Aberrant Splicing

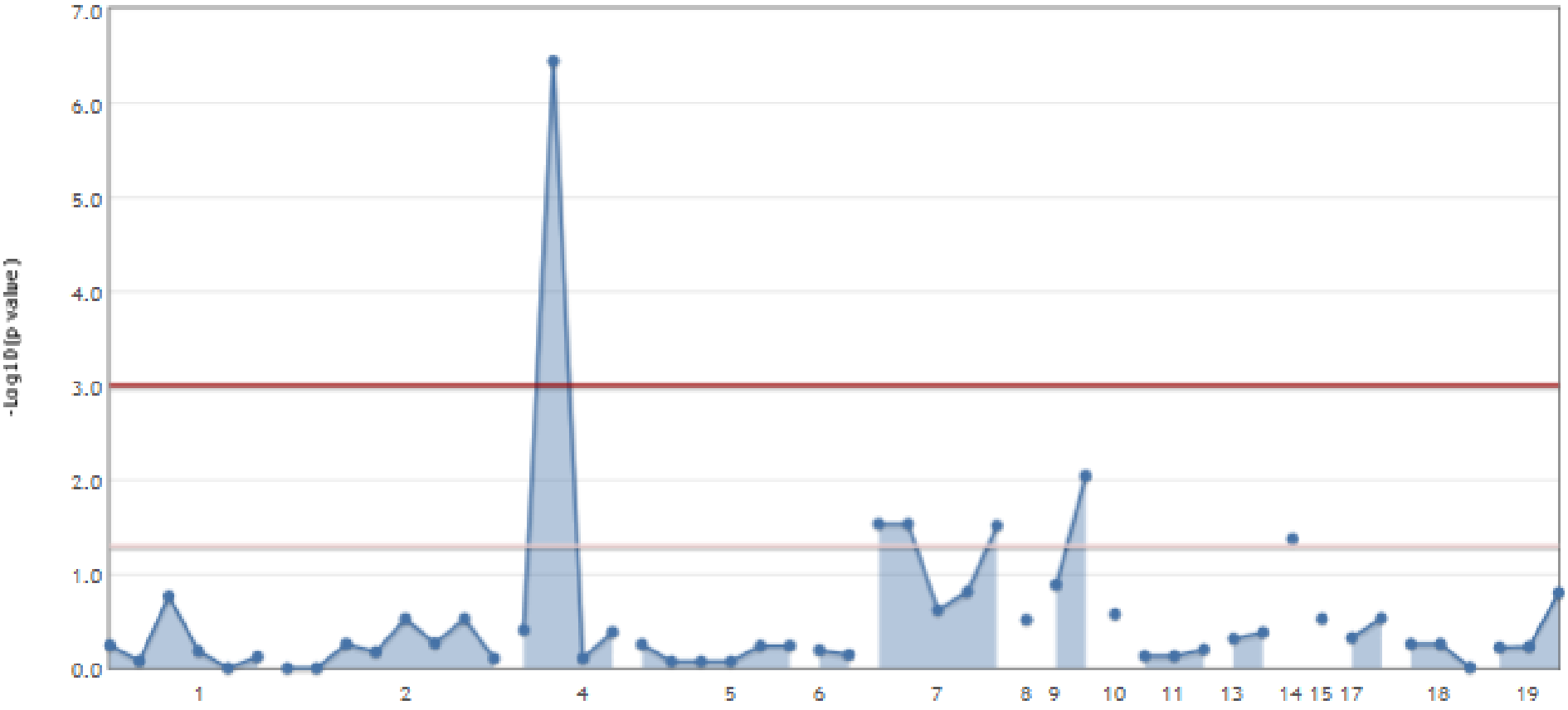


- Exon5 skipping may occur
- Resulting transcript has 123 nt deletion of exon5
- This predicts the in-frame deletion of 41 amino acids beginning after amino acid 178 of the protein, which is normally 358 amino acids long
- Primer set for genotyping the mutation:
  PCR-f: 5'-GCTGAACACACATGGTCCTCTTTCTTG-3'; PCR-r: 5'-GCATTAAGTGCCAACCTCCTGCAATC-3'
  Seq-f: 5'-gaaaccagaagagggagtcag-3'; Seq-r: 5'-TCCTGCAATCTCAACATTTAAAAAAG-3'
  PCR condition:
  1)95˚C 2:00
  2)95˚C 0:30
  3)56˚C 0:30
  4)72˚C 1:00
  5)repeat steps(2-4) 29x
  6)72˚C 7:00
  7)4˚C ∞

Mht plot: recessive model (raw data wG2)

Single pedigree R0443 tested in FACS CD8+ T cells

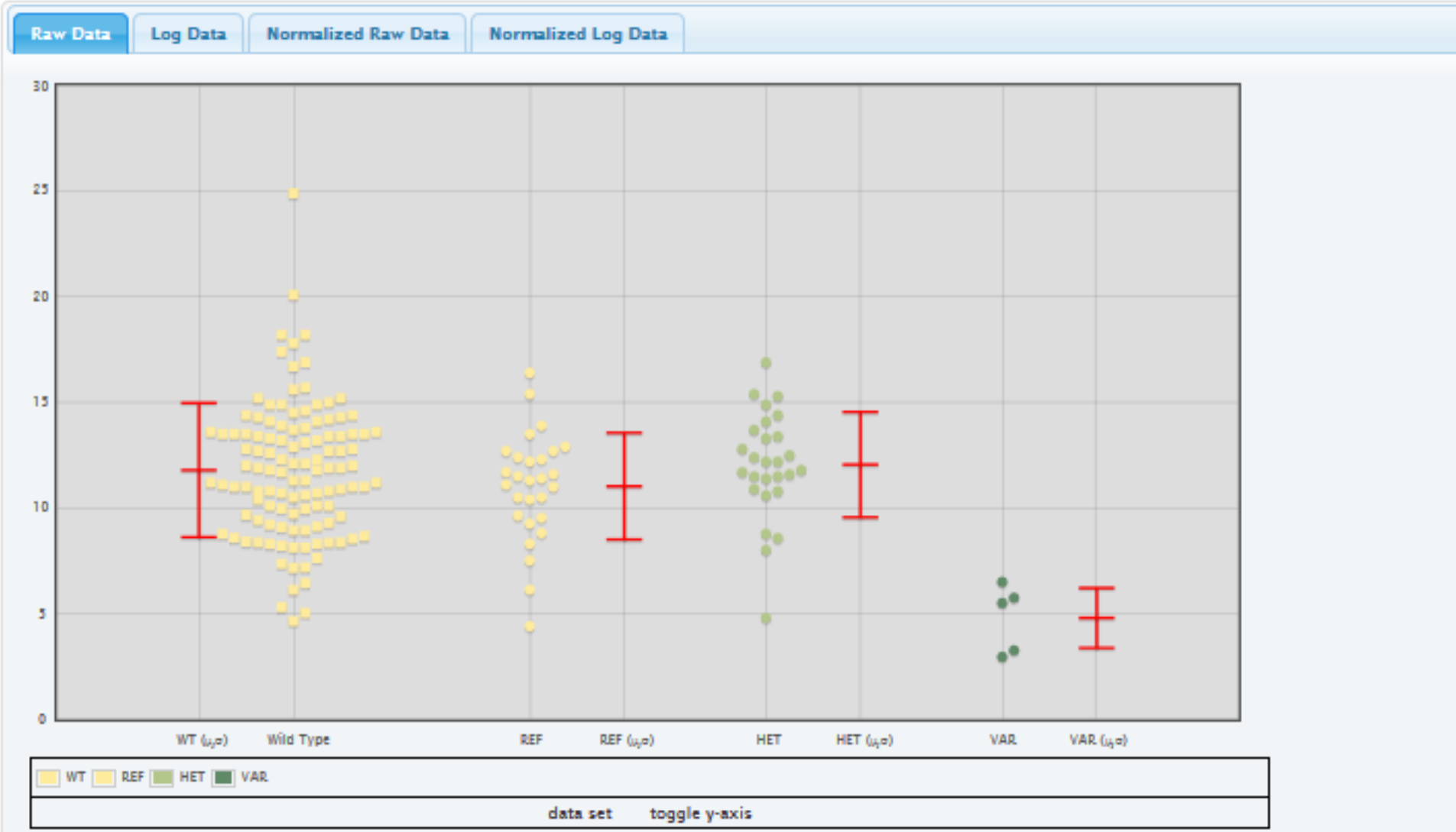
Genotyping Data



FACS CD8+ T cells

Pedigree: R0443

Call: chr4_130378043 (Snrnp40)



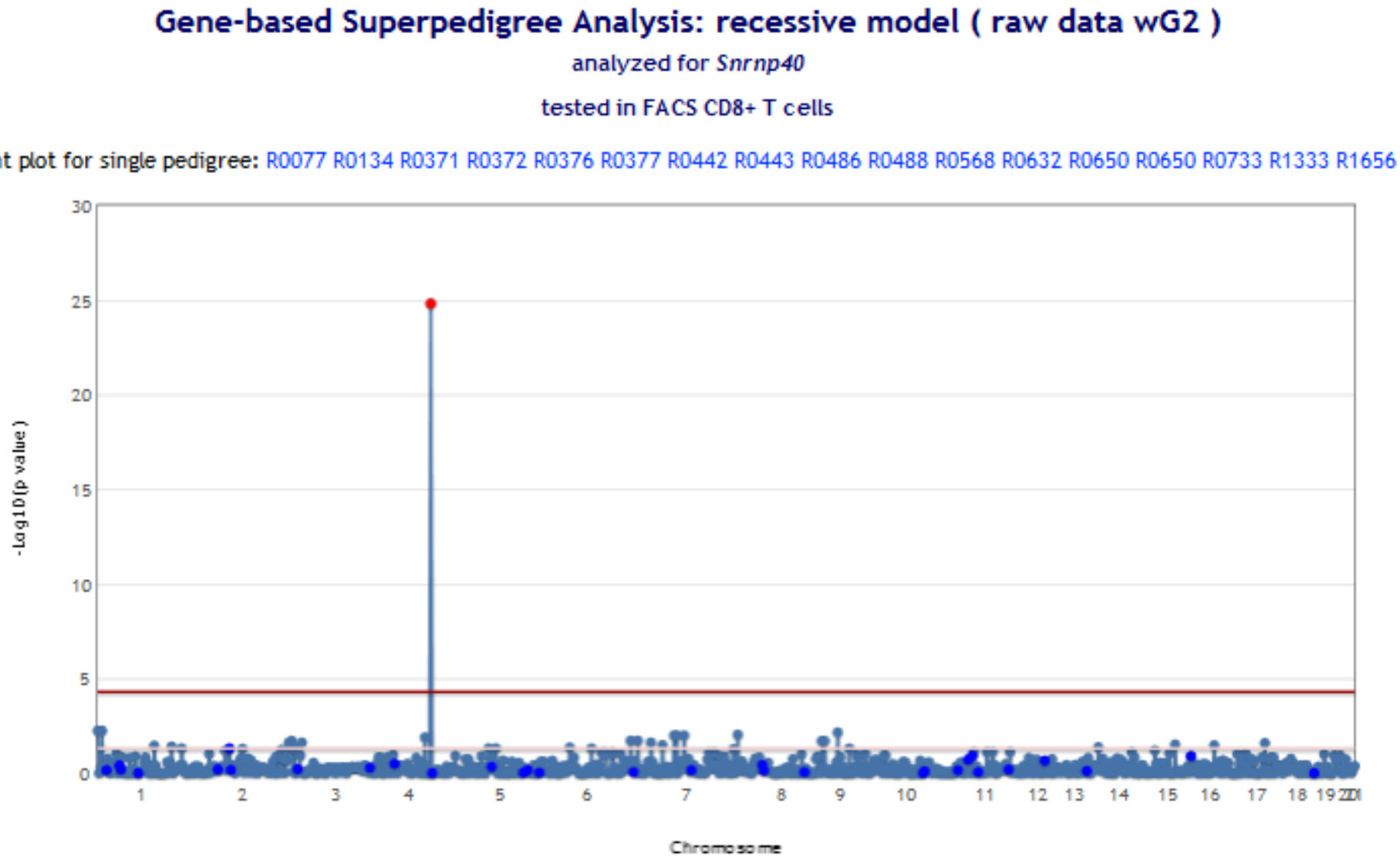
Gradually, much higher resolution is achieved as multiple pedigrees with identical or allelic mutations accumulate in the database

- The computer automatically generates “superpedigrees” when this occurs.
- Eventually, *all* mutations will be incorporated into superpedigrees. Presently 14,272 genes (57% of all genes) have superpedigrees.
- With multiple alleles, confidence in association between phenotype and genotype grows.

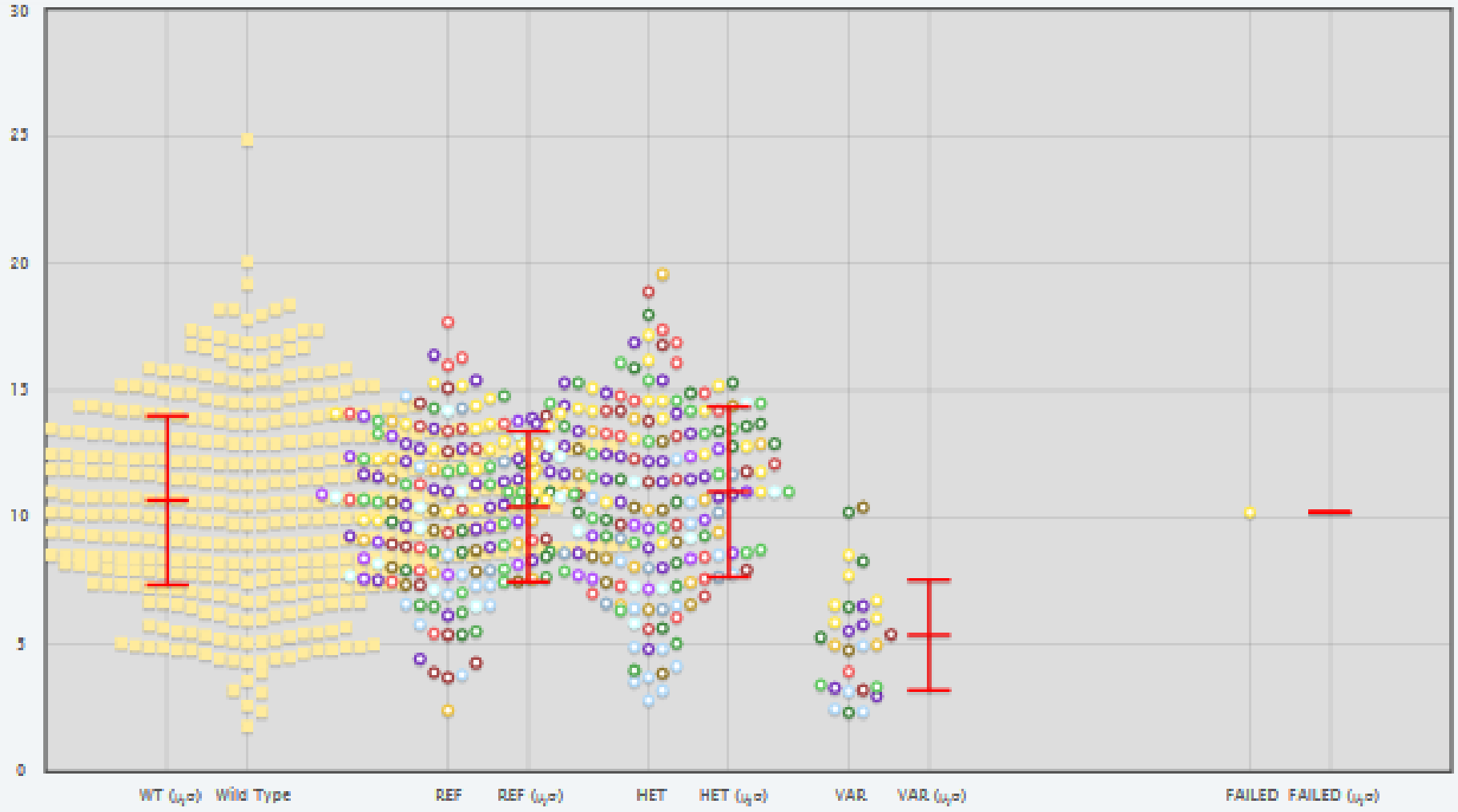
16 pedigrees have the same allele; three other “probably damaging” alleles also exist.

36	Snrnp40	4	1	16	Probably null		FACS CD44+ T MFI	142	
37	Snrnp40	4	4	19	Null + Missense	0.980	FACS CD44+ T MFI	171	
38	Snrnp40	4	4	19	Null + Missense	0.980	FACS CD8+ T cells	207	
39	Snrnp40	4	4	19	Null + Missense	0.980	FACS CD8+ T cells	207	
40	Snrnp40	4	4	19	Null + Missense	0.980	FACS CD8+ T cells	207	
41	Snrnp40	4	1	16	Probably null		FACS CD8+ T cells	151	
42	Snrnp40	4	1	16	Probably null		FACS CD8+ T cells	151	
43	Snrnp40	4	1	16	Probably null		FACS CD8+ T cells	151	
44	Snrnp40	4	4	19	Null + Missense	0.980	FACS CD8+ T cells	207	
45	Snrnp40	4	4	19	Null + Missense	0.980	FACS CD8+ T cells	207	
46	Snrnp40	4	4	19	Null + Missense	0.980	FACS CD8+ T cells	207	
47	Snrnp40	4	1	16	Probably null		FACS CD8+ T cells	151	
48	Snrnp40	4	1	16	Probably null		FACS Macrophages	151	
49	Snrnp40	4	1	16	Probably null		FACS Macrophages	151	

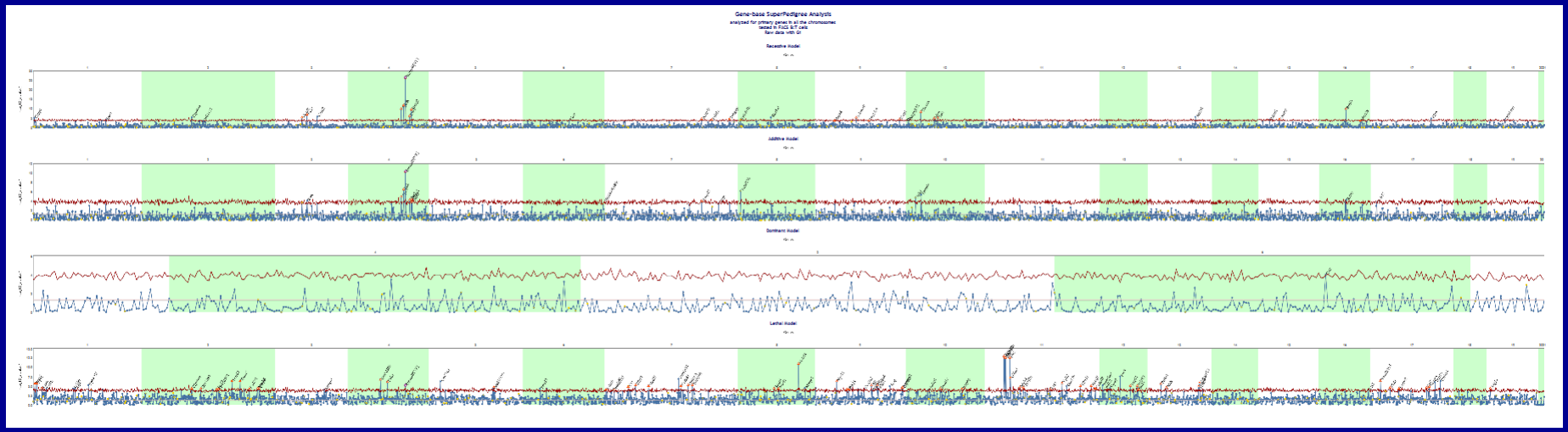
Mapping on 376 G3 mice from 16 pedigrees



Raw Data | Log Data | Normalized Raw Data | Normalized Log Data



Superpedigrees that score in a single screen: whole genome view



B:T cell ratio in peripheral blood: superpedigrees showing inheritance by recessive, additive, and dominant models (top, middle, bottom)

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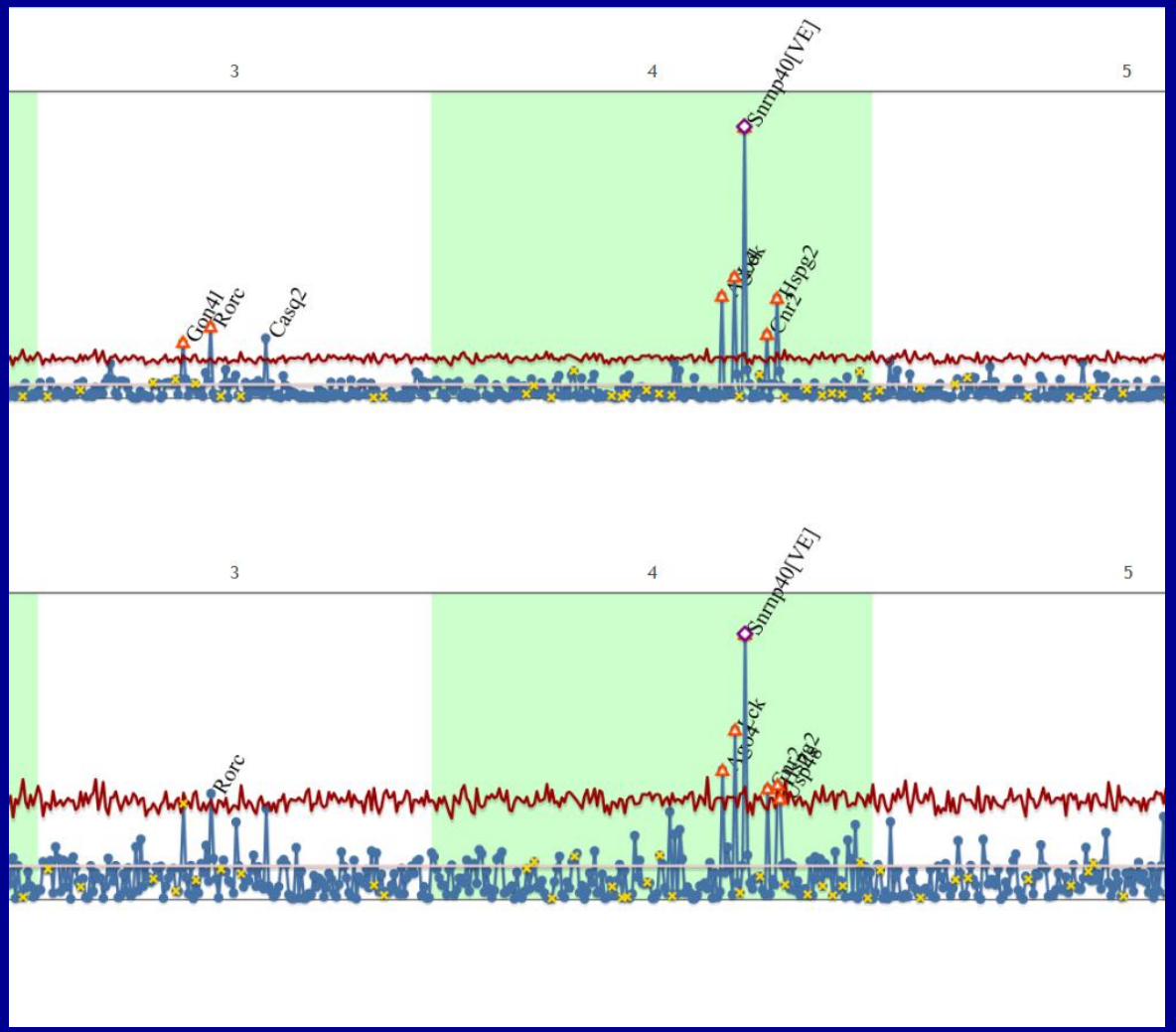
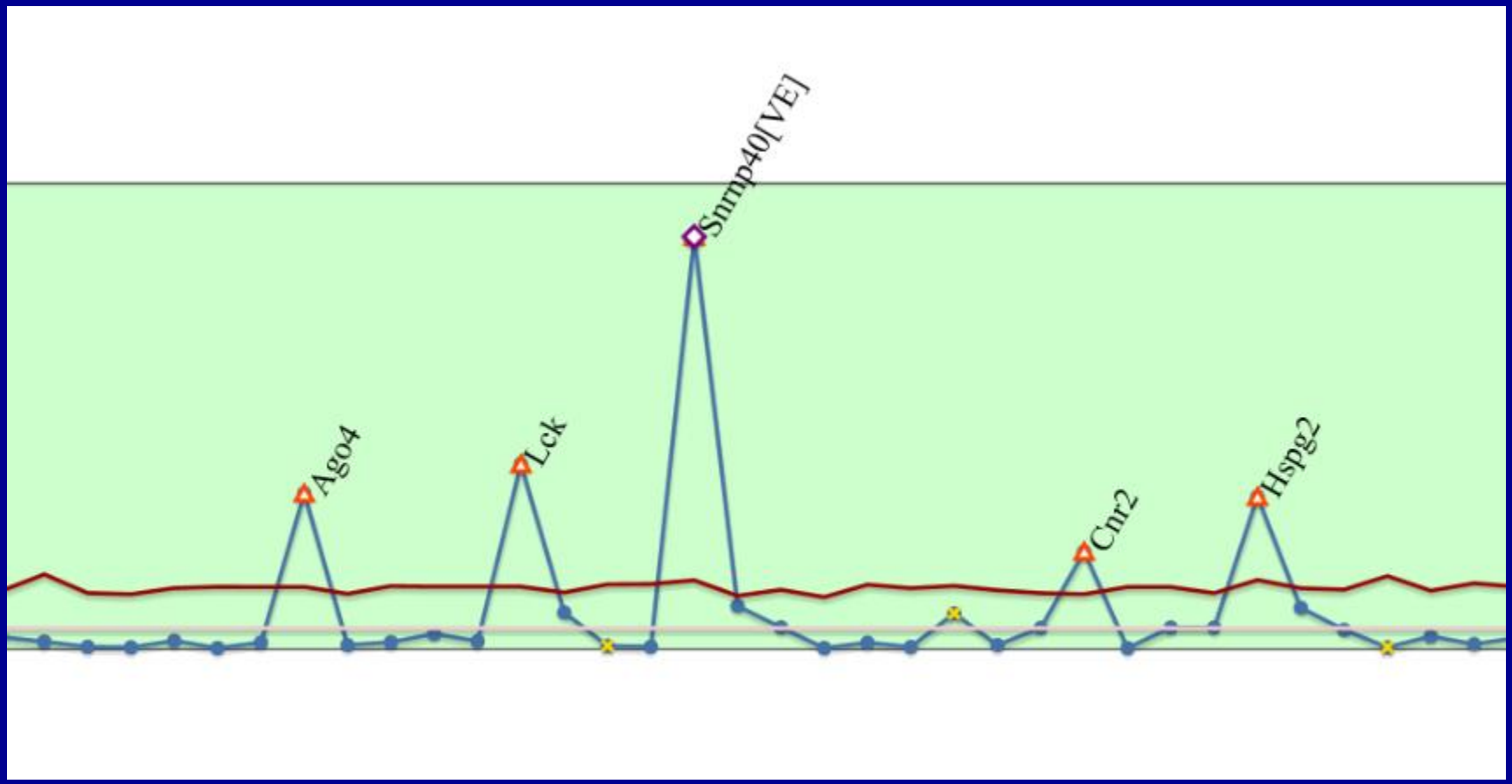
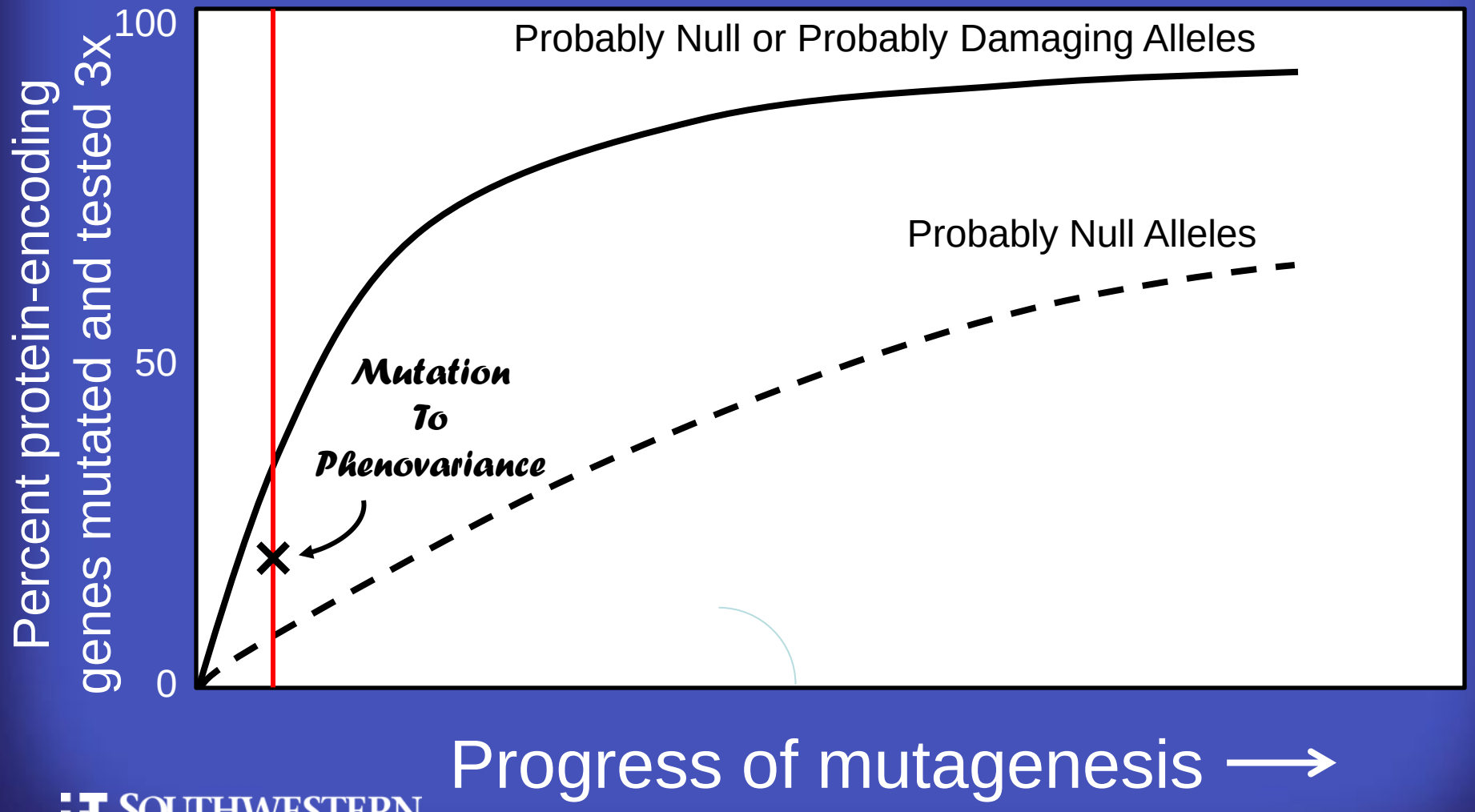
The plot can be enlarged…



…and expanded horizontally for clarity. And as causation is confirmed by CRISPR analysis, individual mutations are marked with purple diamonds



Upper and lower estimates of gene damage will converge over time.



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Real-time identification of causative mutations

- Then: 5 years. Now: 1 hour.
- Then: one phenotype solved in 5 years. Now: 1-2 phenotypes solved per day (~3,000 times as fast as before).
- We are now limited only by the rate at which mutations can be produced and screened: about 600 G3 mice and 1,400 mutations can be analyzed per week, and many of them (0.5% to 1%) cause phenotype detected in our screens.

Real-time identification of causative mutations

- We project the destruction of the majority of genes and analysis of the phenotypic consequences within about three years.
- We will then know most of the genes required for immunity to operate as it does.
- But… establishing mechanism is the real bottleneck.
- Then again… this is an enviable position to occupy.

Betsy Layton

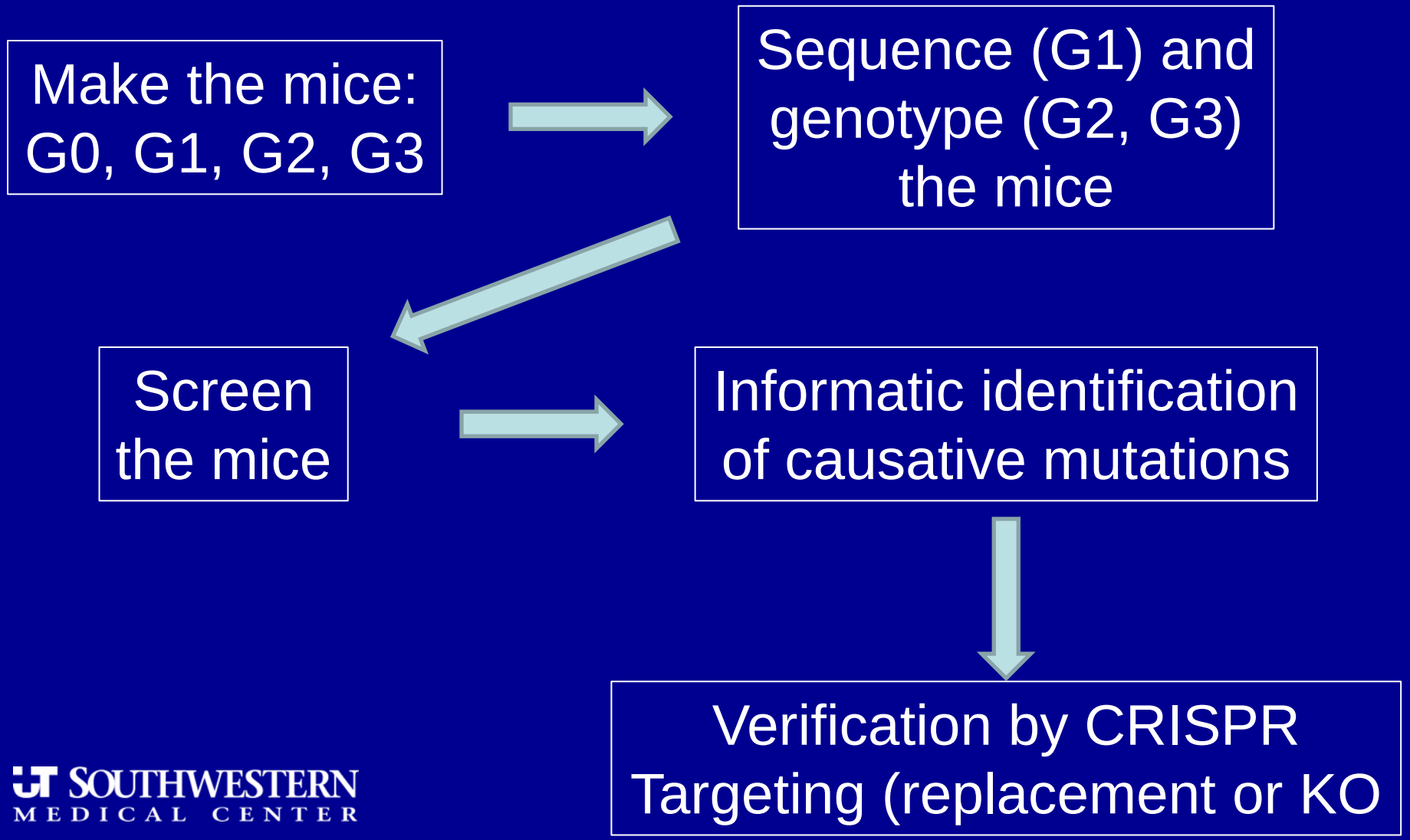
Alexander Poltorak

Christoph Van Huffel

Irina Smirnova

UT SOUTHWESTERN MEDICAL CENTER

Five essential processes are needed for high speed positional cloning



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