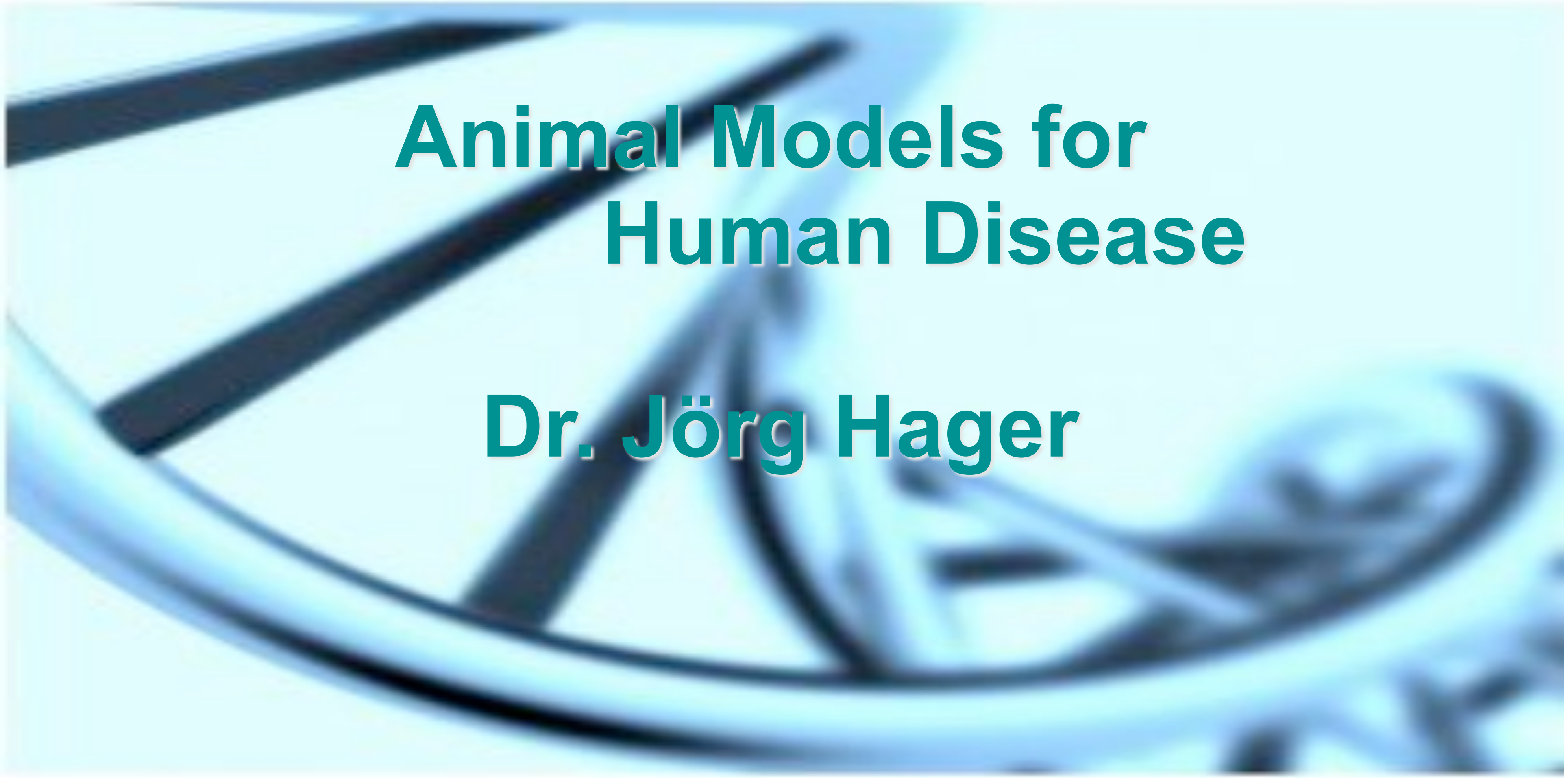
cea

C N G



Animal Models for Human Disease

Dr. Jörg Hager

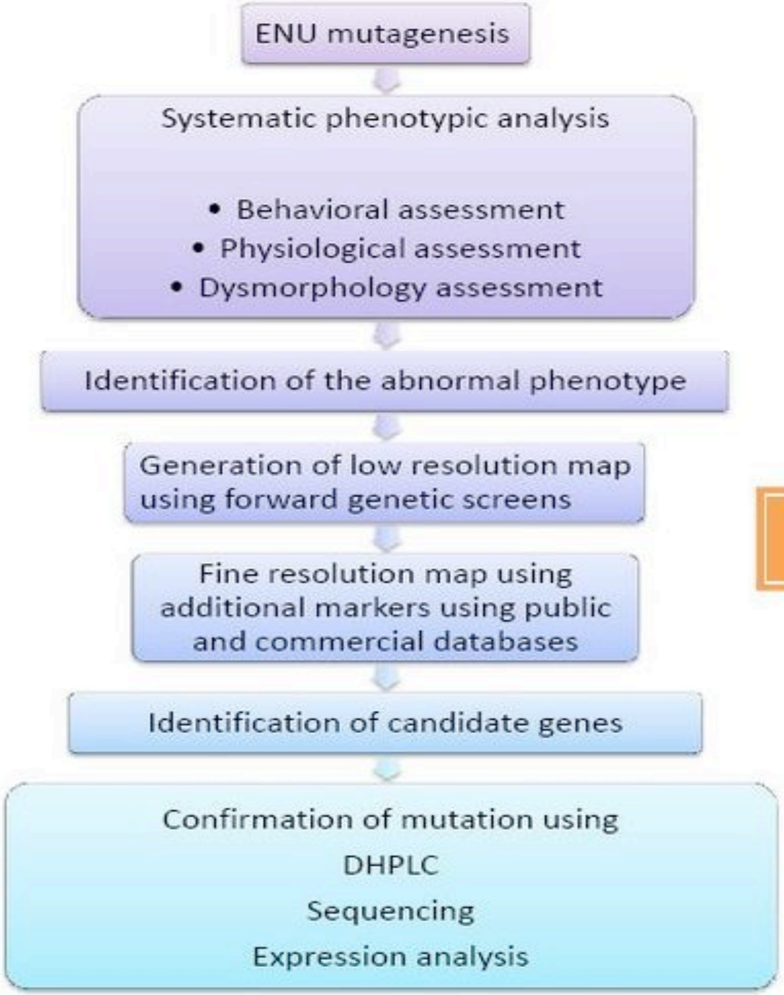
Understanding gene function through ENU mutagenesis

CNG

- ENU, (*N-ethyl-N-nitrosourea* ($C_3H_7N_3O_2$)), is a highly potent mutagen.
- ENU induces about 1point-mutation every 1 to 2 megabases.
- ENU targets spermatogonial stem cells
- ENU induced mutations include loss-of-function as well as gain-of-function mutations.

Classical Workflow for ENU mutant screen

C N G



ENU mutagenesis

Systematic phenotypic analysis

- Behavioral assessment
- Physiological assessment
- Dysmorphology assessment

Identification of the abnormal phenotype

Generation of low resolution map using forward genetic screens

Fine resolution map using additional markers using public and commercial databases

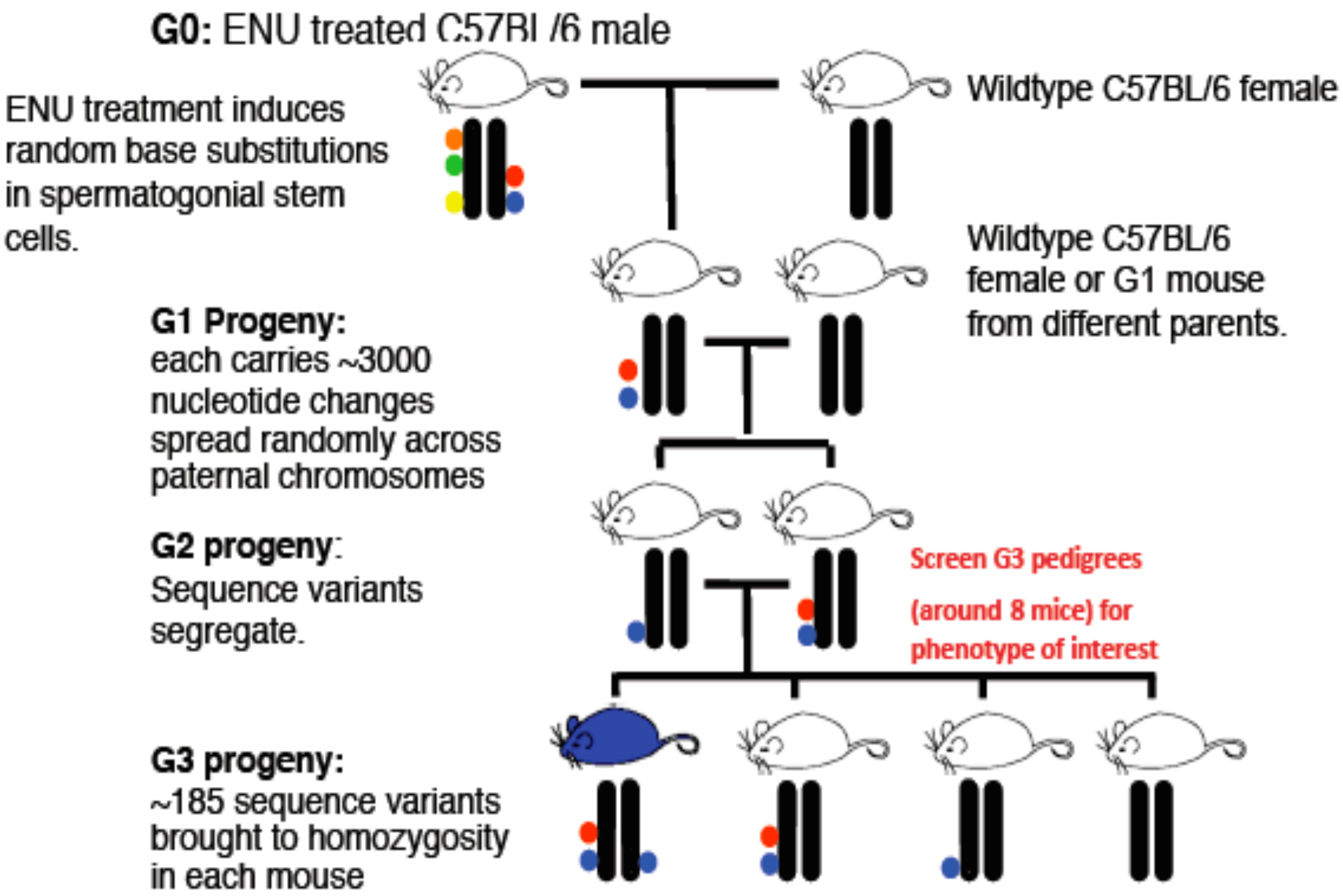
Identification of candidate genes

Confirmation of mutation using
DHPLC
Sequencing
Expression analysis

Figure 1: Overview of ENU mutagenesis screen

Adapted from: Hans Hedrich (editor). The Laboratory Mouse, Elsevier Academic Press. ISBN 0-12-336425-6

ENU Mutagenesis



G0: ENU treated C57BL/6 male

ENU treatment induces random base substitutions in spermatogonial stem cells.

Wildtype C57BL/6 female

Wildtype C57BL/6 female or G1 mouse from different parents.

G1 Progeny:
each carries ~3000 nucleotide changes spread randomly across paternal chromosomes

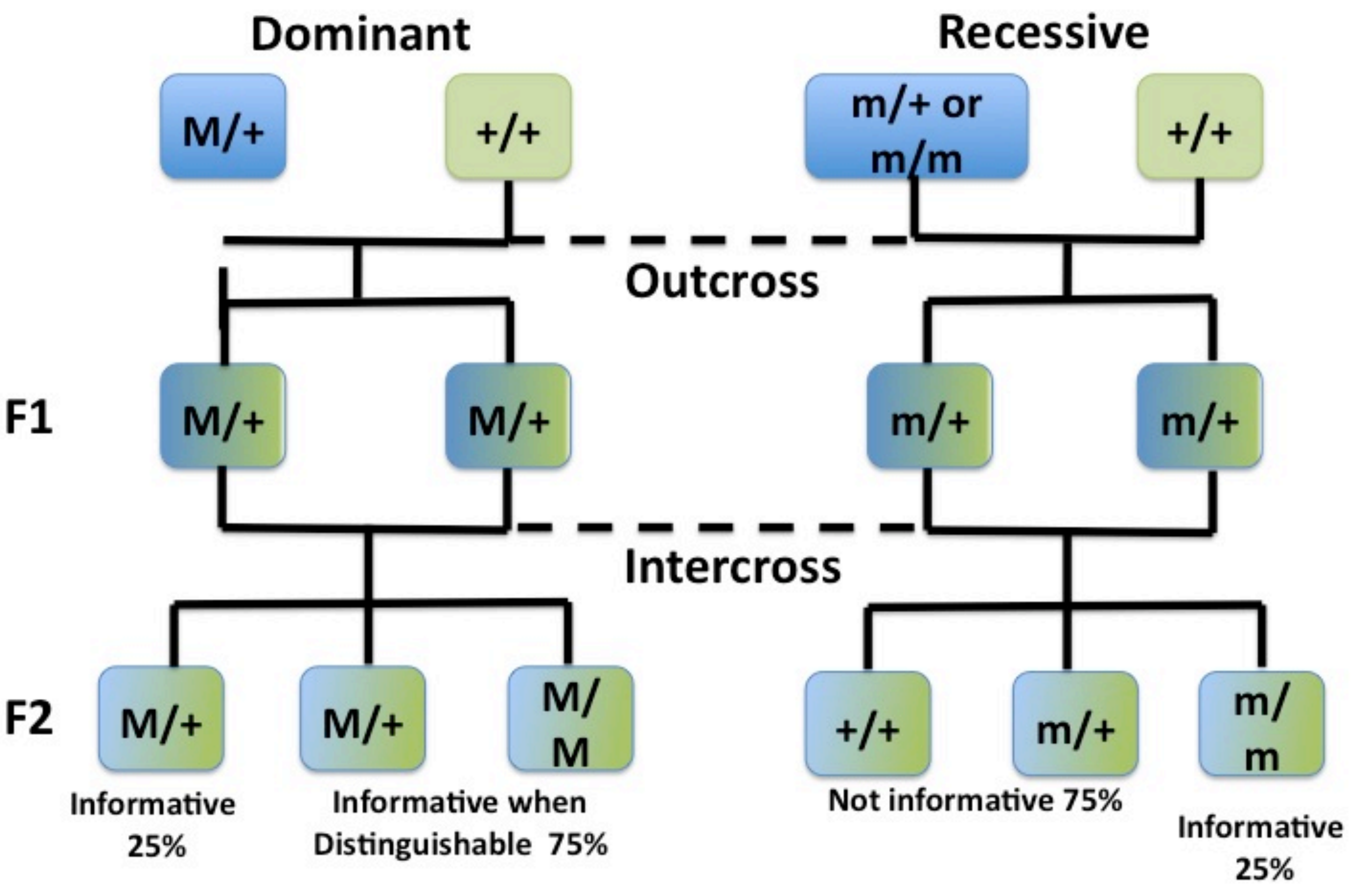
G2 progeny:
Sequence variants segregate.

Screen G3 pedigrees (around 8 mice) for phenotype of interest

G3 progeny:
~185 sequence variants brought to homozygosity in each mouse

Courtesy of: Anselm Enders, ANU

Breeding scheme for positional cloning



Adapted from: Hans Hedrich (editor). The Laboratory Mouse, Elsevier Academic Press. ISBN 0-12-336425-6

Positional cloning necessitates large scale animal breeding

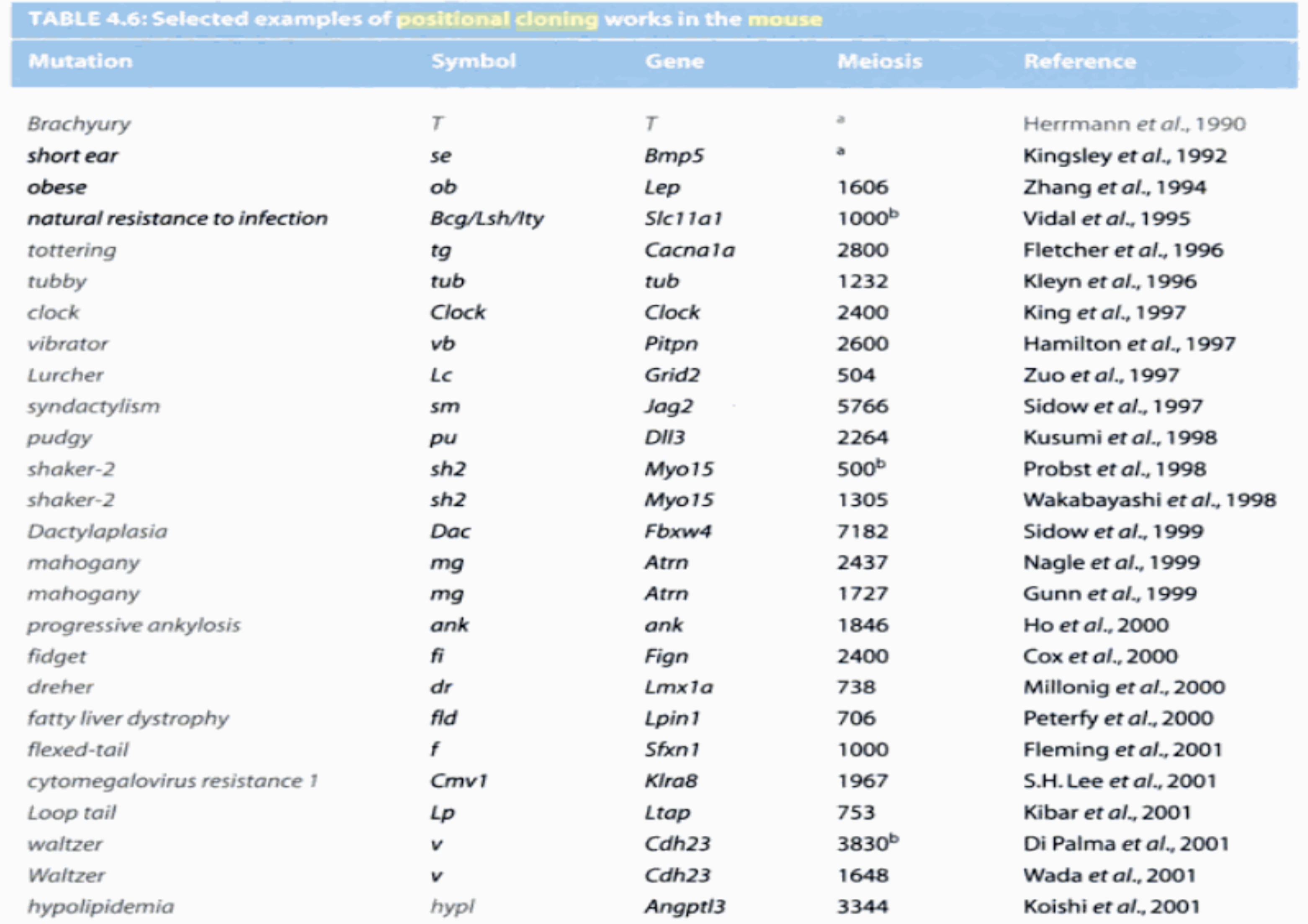
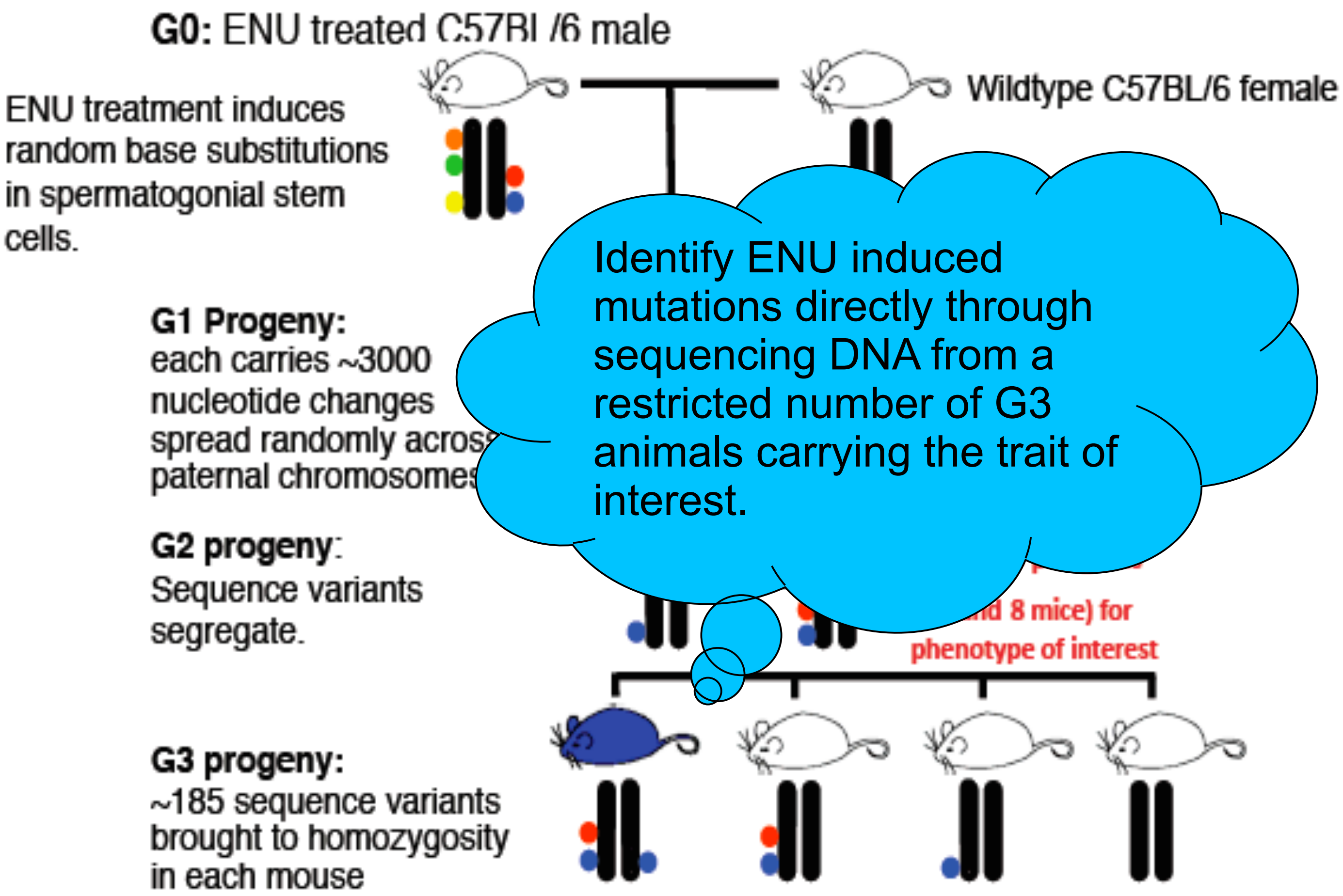


TABLE 4.6: Selected examples of positional cloning works in the mouse

Mutation	Symbol	Gene	Meiosis	Reference
Brachyury	*T*	*T*	[a]	Herrmann *et al.*, 1990
short ear	*se*	*Bmp5*	[a]	Kingsley *et al.*, 1992
obese	*ob*	*Lep*	1606	Zhang *et al.*, 1994
natural resistance to infection	*Bcg/Lsh/Ity*	*Slc11a1*	1000[b]	Vidal *et al.*, 1995
tottering	*tg*	*Cacna1a*	2800	Fletcher *et al.*, 1996
tubby	*tub*	*tub*	1232	Kleyn *et al.*, 1996
clock	*Clock*	*Clock*	2400	King *et al.*, 1997
vibrator	*vb*	*Pitpn*	2600	Hamilton *et al.*, 1997
Lurcher	*Lc*	*Grid2*	504	Zuo *et al.*, 1997
syndactylism	*sm*	*Jag2*	5766	Sidow *et al.*, 1997
pudgy	*pu*	*Dll3*	2264	Kusumi *et al.*, 1998
shaker-2	*sh2*	*Myo15*	500[b]	Probst *et al.*, 1998
shaker-2	*sh2*	*Myo15*	1305	Wakabayashi *et al.*, 1998
Dactylaplasia	*Dac*	*Fbxw4*	7182	Sidow *et al.*, 1999
mahogany	*mg*	*Atrn*	2437	Nagle *et al.*, 1999
mahogany	*mg*	*Atrn*	1727	Gunn *et al.*, 1999
progressive ankylosis	*ank*	*ank*	1846	Ho *et al.*, 2000
fidget	*fi*	*Fign*	2400	Cox *et al.*, 2000
dreher	*dr*	*Lmx1a*	738	Millonig *et al.*, 2000
fatty liver dystrophy	*fld*	*Lpin1*	706	Peterfy *et al.*, 2000
flexed-tail	*f*	*Sfxn1*	1000	Fleming *et al.*, 2001
cytomegalovirus resistance 1	*Cmv1*	*Klra8*	1967	S.H. Lee *et al.*, 2001
Loop tail	*Lp*	*Ltap*	753	Kibar *et al.*, 2001
waltzer	*v*	*Cdh23*	3830[b]	Di Palma *et al.*, 2001
Waltzer	*v*	*Cdh23*	1648	Wada *et al.*, 2001
hypolipidemia	*hypl*	*Angptl3*	3344	Koishi *et al.*, 2001

Adapted from: Hans Hedrich (editor). The Laboratory Mouse, Elsevier Academic Press. ISBN 0-12-336425-6

ENU Mutagenesis: Shortcut through sequencing



G0: ENU treated C57BL/6 male

Wildtype C57BL/6 female

ENU treatment induces random base substitutions in spermatogonial stem cells.

G1 Progeny:
each carries ~3000 nucleotide changes spread randomly across paternal chromosomes

G2 progeny:
Sequence variants segregate.

G3 progeny:
~185 sequence variants brought to homozygosity in each mouse

nd 8 mice) for phenotype of interest

Identify ENU induced mutations directly through sequencing DNA from a restricted number of G3 animals carrying the trait of interest.

ENU mutagenesis project at CNG

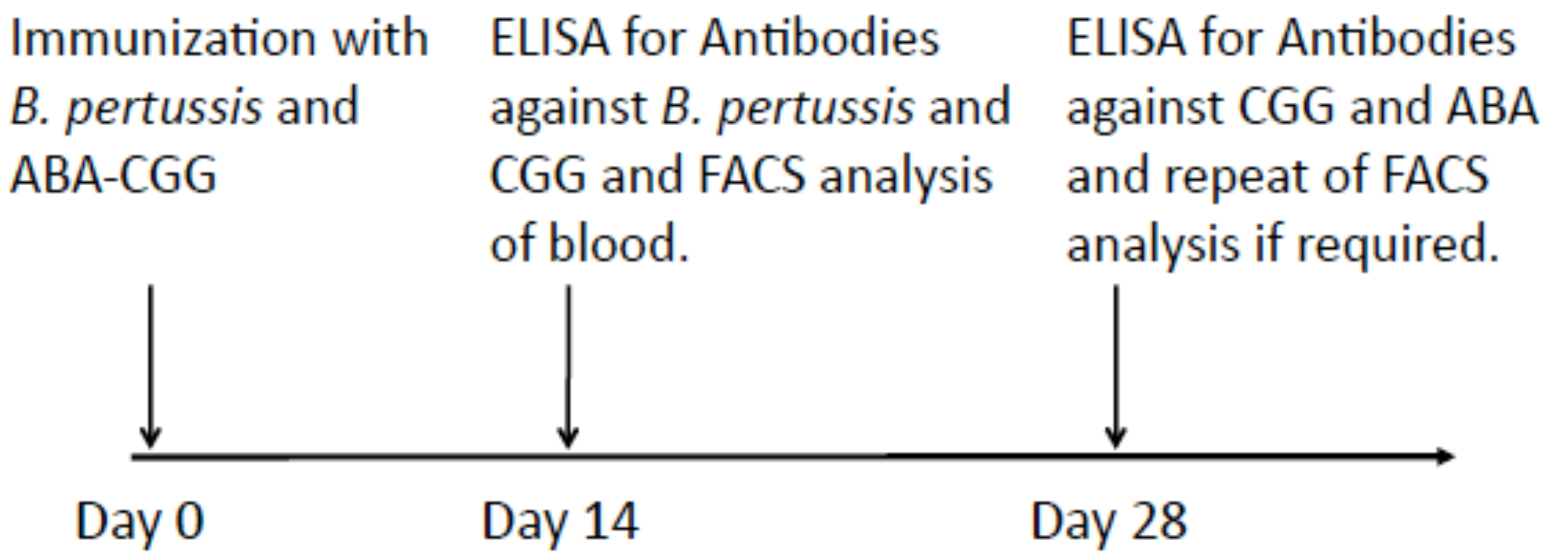
Infection and Immunity Genomics Consortium (IIGC)

Collaboration between the Australian National University (ANU) and CNG

Scope of the project:

- generate a library of new ENU mutants with defects affecting autoimmunity, vaccination and immunity to tuberculosis infection
- develop ways to refine phenotype screens and accelerate the identification of mutations.

Immunization and FACS screen



Immunization with *B. pertussis* and ABA-CGG

Day 0

ELISA for Antibodies against *B. pertussis* and CGG and FACS analysis of blood.

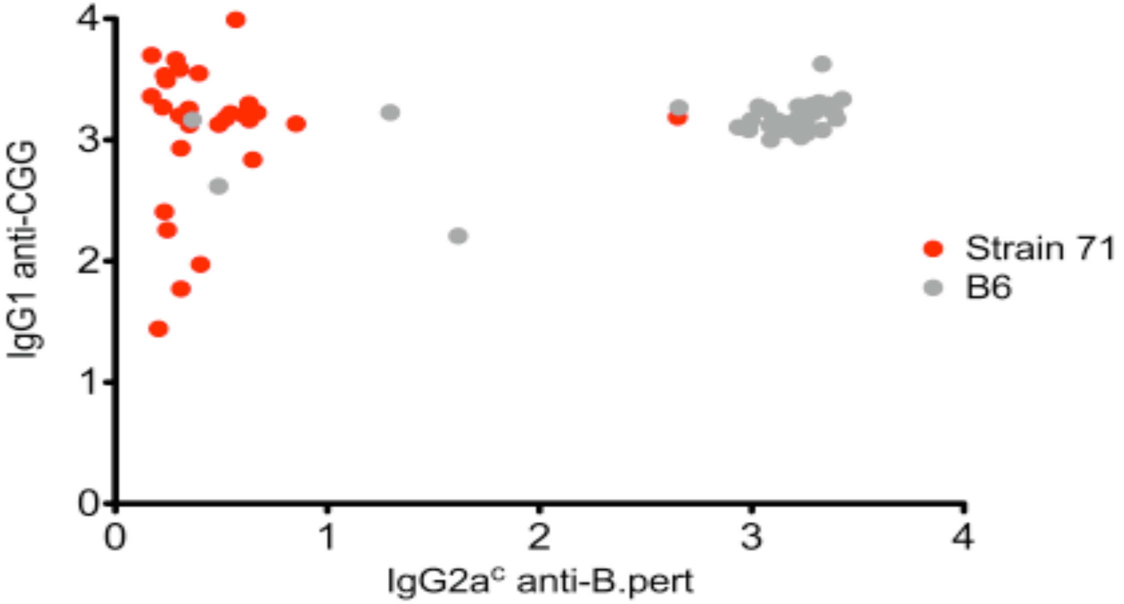
Day 14

ELISA for Antibodies against CGG and ABA and repeat of FACS analysis if required.

Day 28

B. pertussis induces a Th1-dependent response
CGG induces a Th2-dependent result
The response to the hapten arsonate requires a germinal centre reaction.

Immunization screen: Example 1 ENU7B6 71

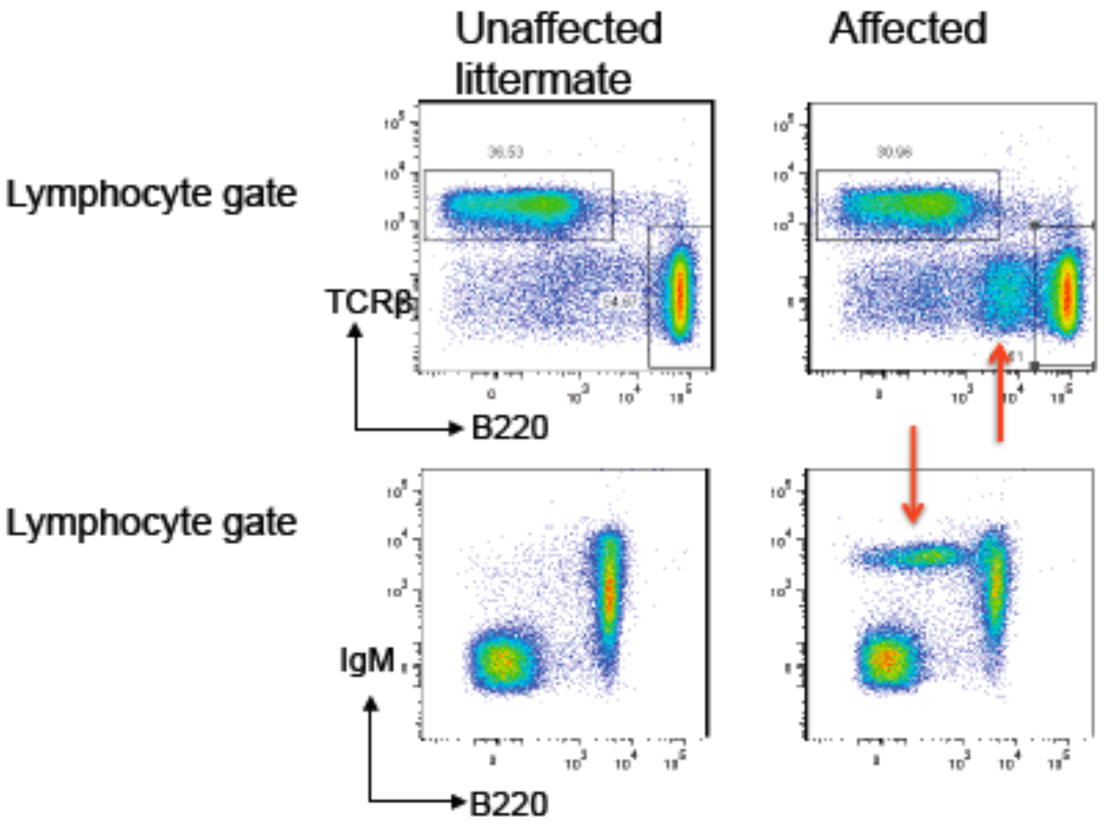


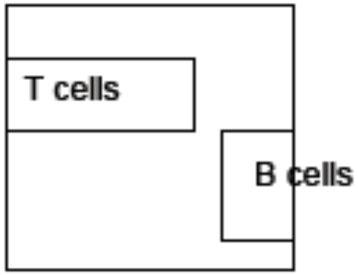
Phenotype:
Low Th1-dependent antibody response to *Bordetella pertussis* but good response to chicken Y-globulin.
No abnormalities in the development of T or B cells detected.

Immunization screen: Example 2 ENU6WTNIH33

C N G

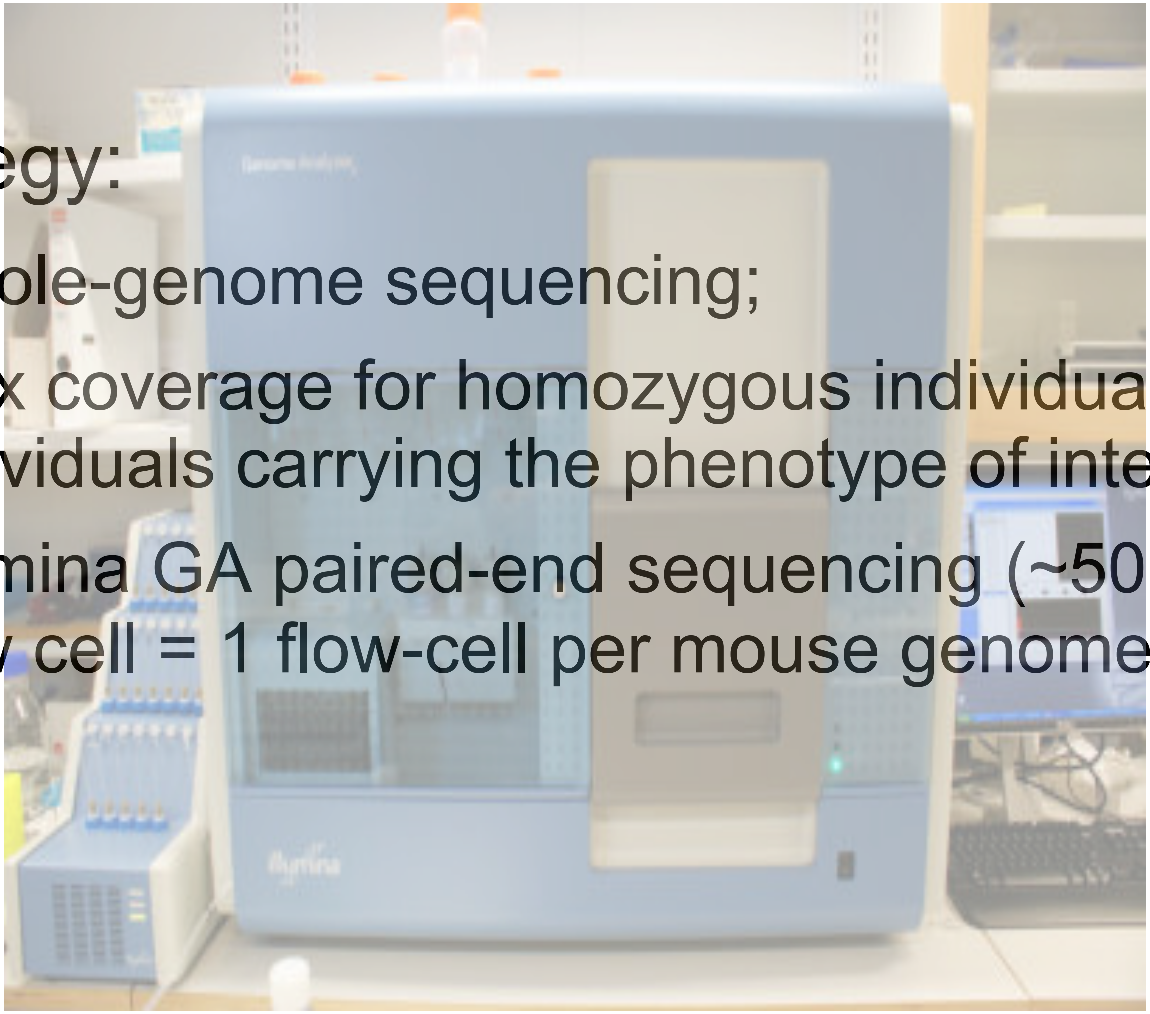
Phenotype:
Development of B cell lymphomas, in some cases T cell lymphomas.





Tumors are B220lo and positive for IgM, IgD, CD19 and CD5.

Identifiction of mutations by HT sequencing



- Strategy:
  - Whole-genome sequencing;
  - 6-8x coverage for homozygous individuals of G3 individuals carrying the phenotype of interest;
  - Illumina GA paired-end sequencing (~50 Gbases/ flow cell = 1 flow-cell per mouse genome;

CNG Sequencing Pipeline

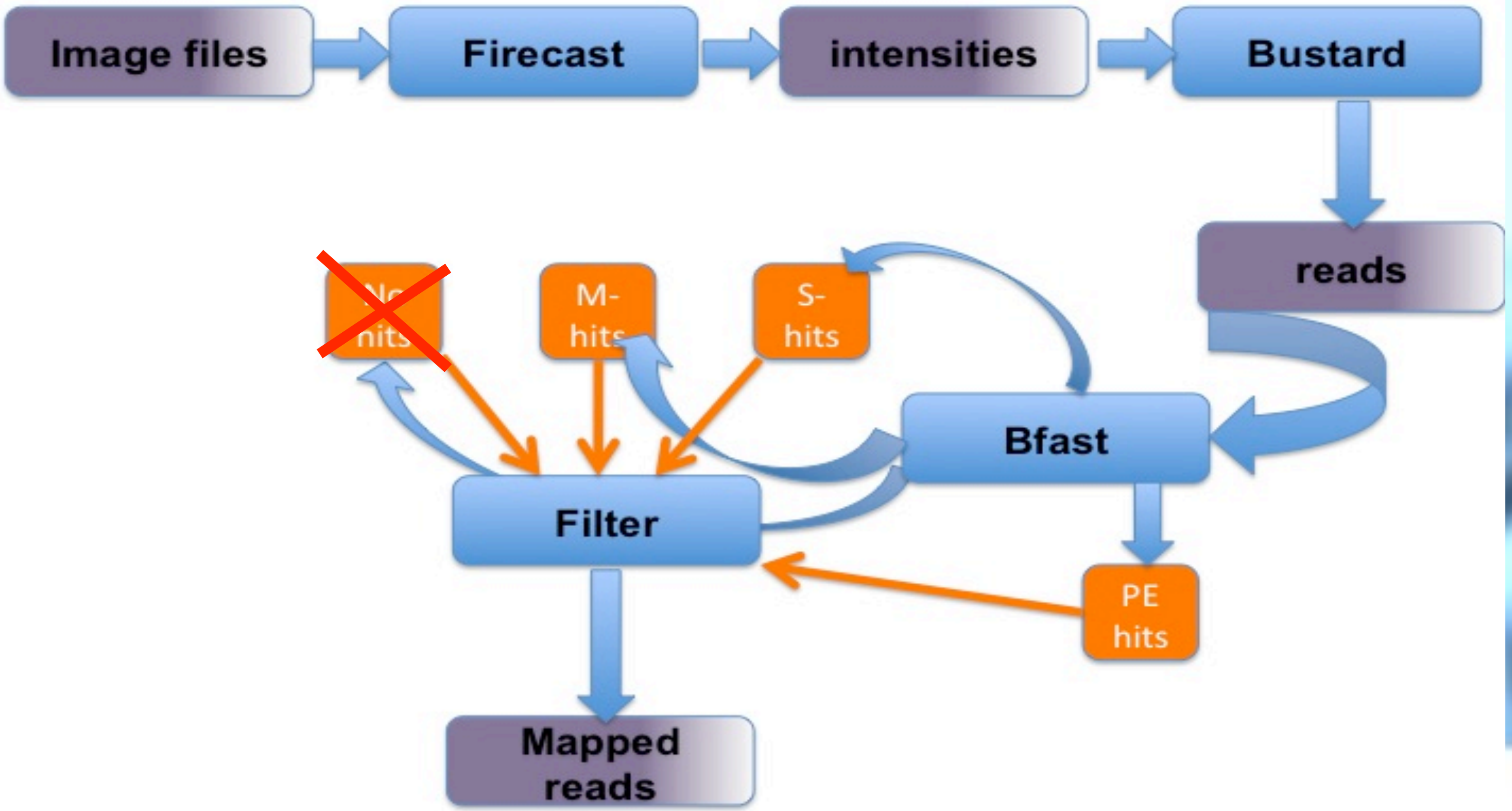


Image files

Firecast

intensities

Bustard

reads

~~No-hits~~

M-hits

S-hits

Bfast

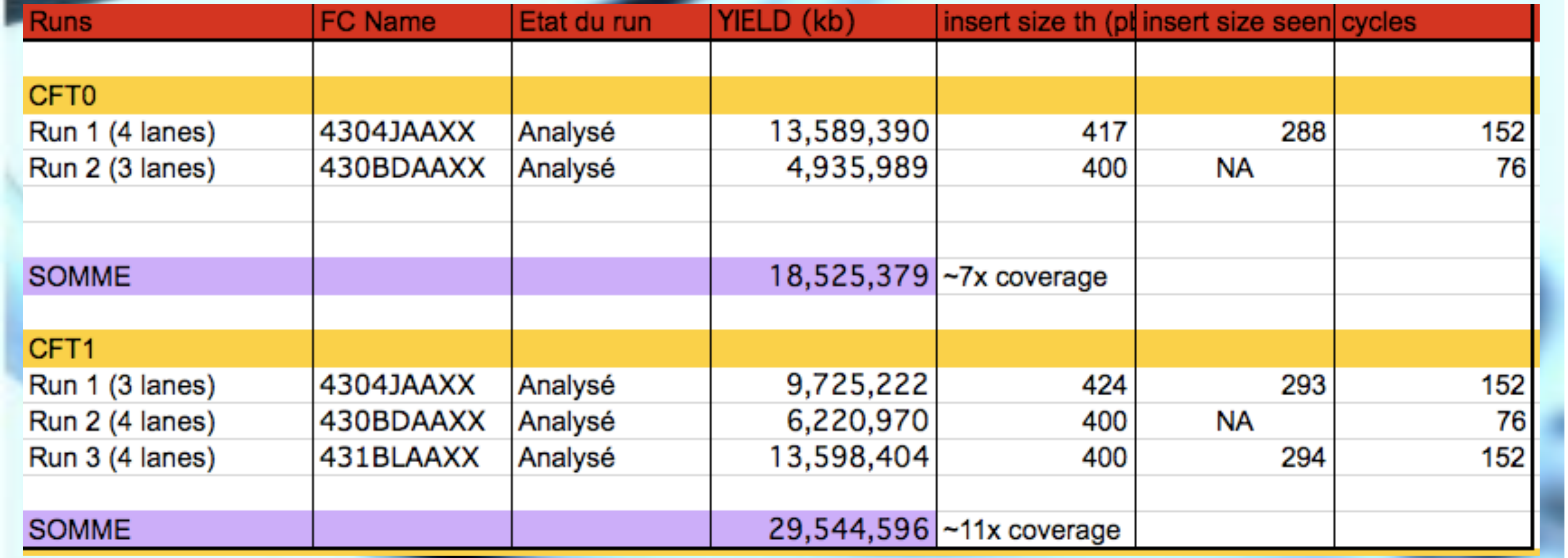
Filter

PE hits

Mapped reads

Schema courtesy of Mario Foglio, CNG

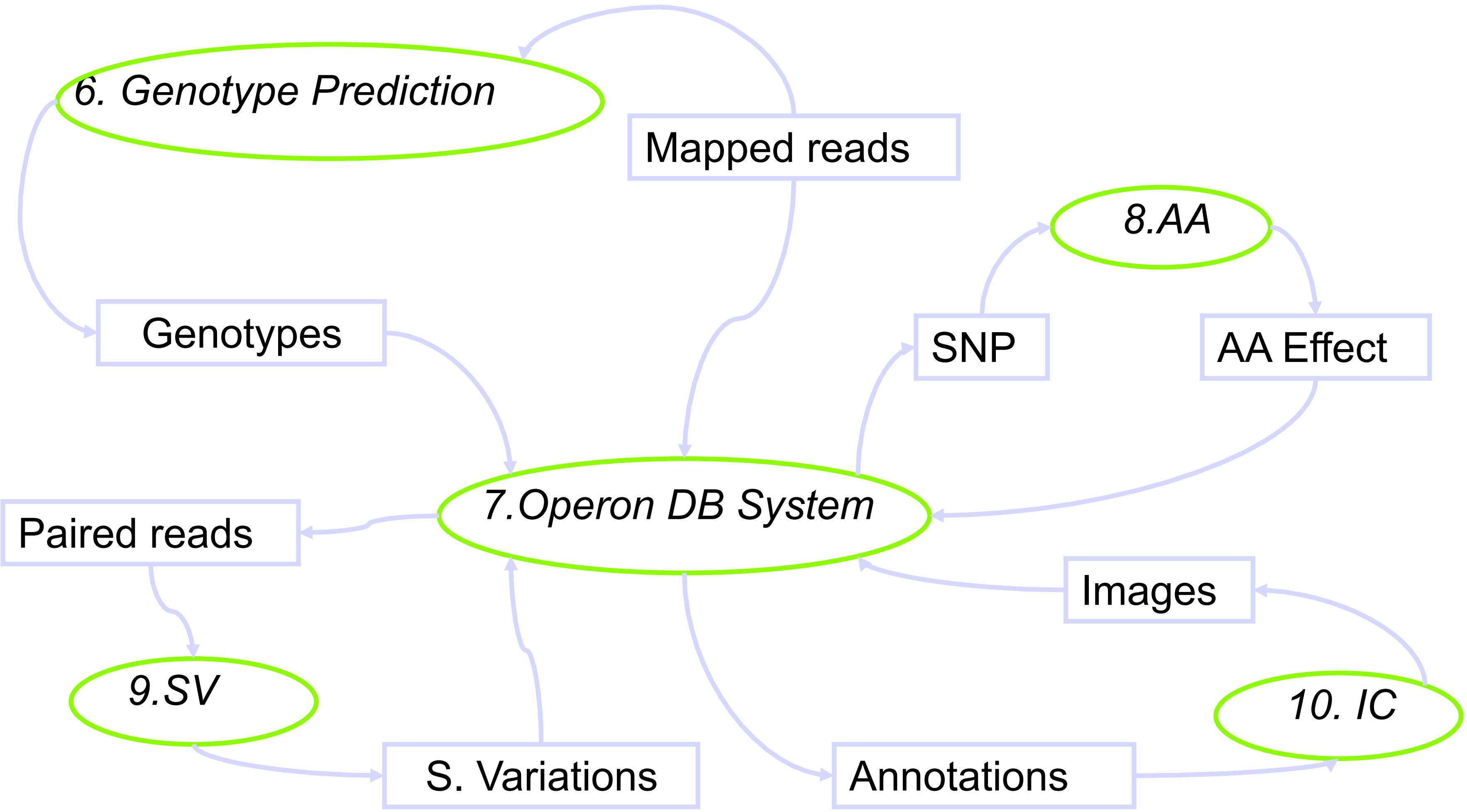
WG Sequence from two ENU mouse strains



Runs	FC Name	Etat du run	YIELD (kb)	insert size th (p	insert size seen	cycles
CFT0						
Run 1 (4 lanes)	4304JAAXX	Analysé	13,589,390	417	288	152
Run 2 (3 lanes)	430BDAAXX	Analysé	4,935,989	400	NA	76
SOMME			18,525,379	~7x coverage		
CFT1						
Run 1 (3 lanes)	4304JAAXX	Analysé	9,725,222	424	293	152
Run 2 (4 lanes)	430BDAAXX	Analysé	6,220,970	400	NA	76
Run 3 (4 lanes)	431BLAAXX	Analysé	13,598,404	400	294	152
SOMME			29,544,596	~11x coverage		

Workflow: Genotyping aligned sequences

C N G



6. Genotype Prediction

Mapped reads

8.AA

Genotypes

SNP

AA Effect

7.Operon DB System

Paired reads

Images

9.SV

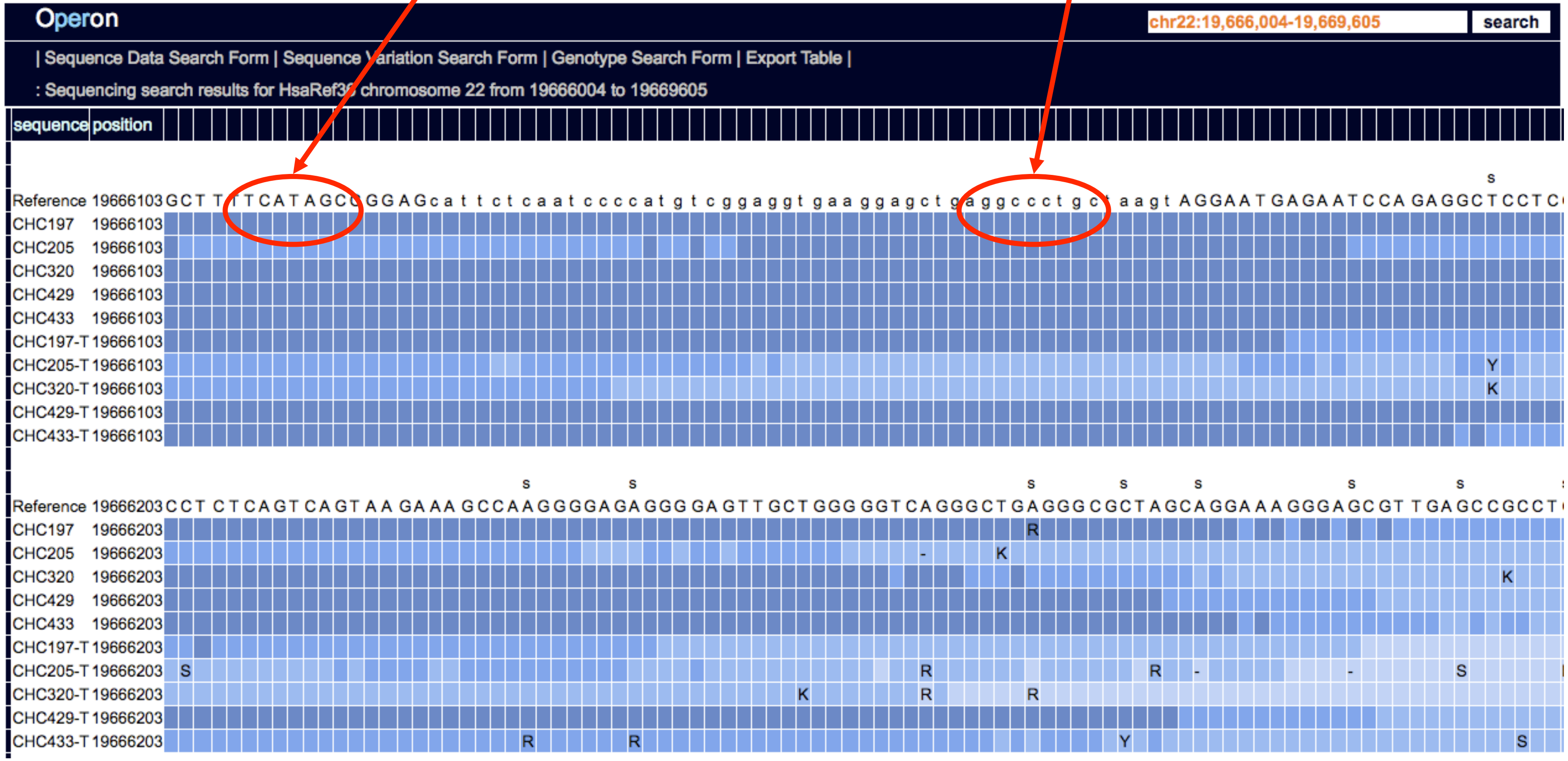
10. IC

S. Variations

Annotations

6 S. Heat; 7 M. Foglio; 8 V. Renault; 10 Z. Wang.

Visualizing aligned sequence quality



Reference

Repeat in lower case

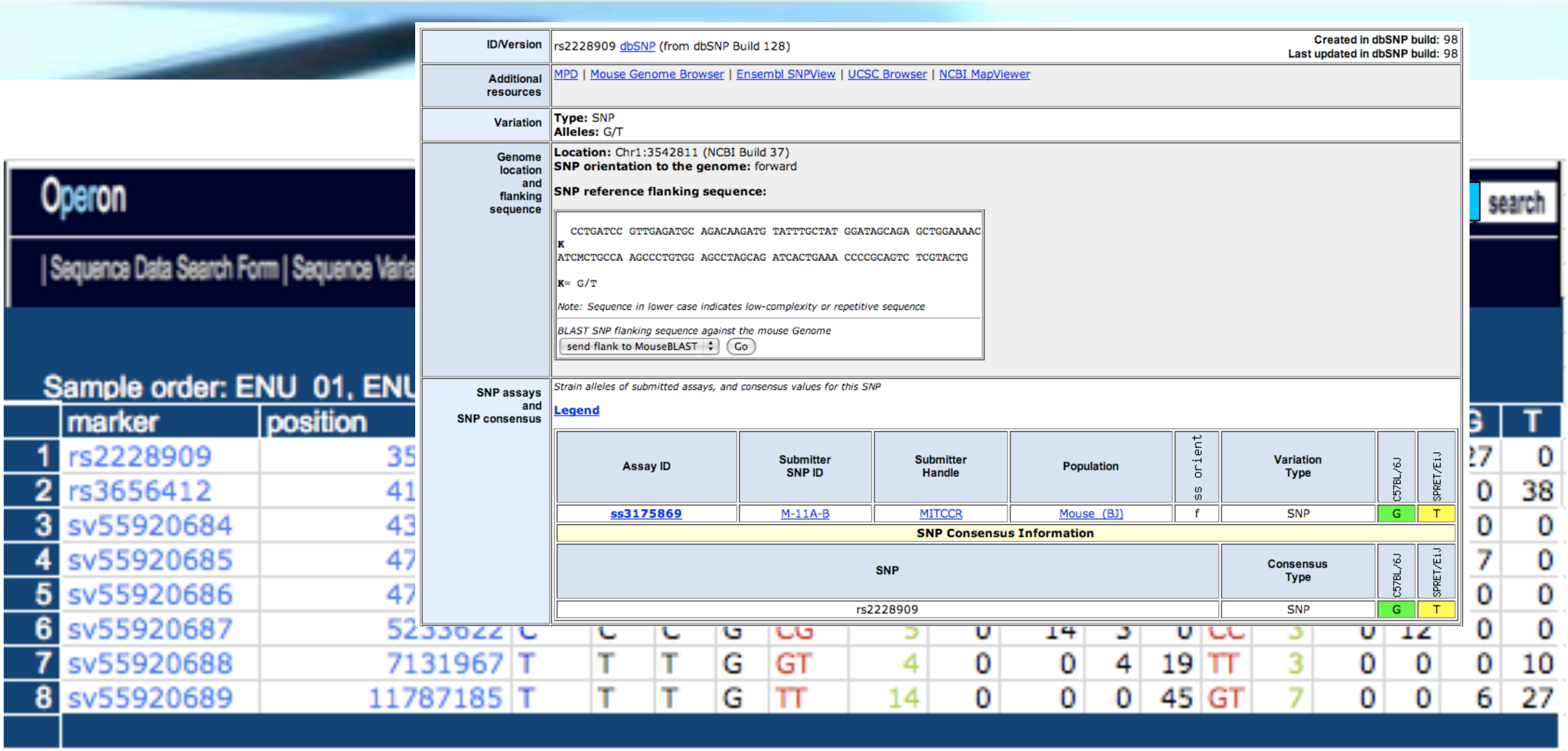
Operon

| Sequence Data Search Form | Sequence Variation Search Form | Genotype Search Form | Export Table |

: Sequencing search results for HsaRef36 chromosome 22 from 19666004 to 19669605

sequence	position	
Reference	19666103	GCTTTTCATAGCCGGAGcattctcaatccccatgtcggaggtgaaggagctgaggccctgctaagtAGGAATGAGAATCCAGAGGCTCCTC
CHC197	19666103	
CHC205	19666103	
CHC320	19666103	
CHC429	19666103	
CHC433	19666103	
CHC197-T	19666103	
CHC205-T	19666103	Y
CHC320-T	19666103	K
CHC429-T	19666103	
CHC433-T	19666103	
Reference	19666203	CCTCTCAGTCAGTAAGAAAGCCAAGGGGAGAGGGGAGTTGCTGGGGGTCAGGGCTGAGGGCGCTAGCAGGAAAGGGAGCGTTGAGCCGCCT
CHC197	19666203	R
CHC205	19666203	- K
CHC320	19666203	K
CHC429	19666203	
CHC433	19666203	
CHC197-T	19666203	
CHC205-T	19666203	S R R - - S
CHC320-T	19666203	K R R
CHC429-T	19666203	
CHC433-T	19666203	R R Y S

Identification of sequence variants in Operon



ID/Version	rs2228909 dbSNP (from dbSNP Build 128) **Created in dbSNP build:** 98 **Last updated in dbSNP build:** 98
Additional resources	MPD | Mouse Genome Browser | Ensembl SNPView | UCSC Browser | NCBI MapViewer
Variation	**Type:** SNP **Alleles:** G/T
Genome location and flanking sequence	**Location:** Chr1:3542811 (NCBI Build 37) **SNP orientation to the genome:** forward **SNP reference flanking sequence:**
SNP assays and SNP consensus	*Strain alleles of submitted assays, and consensus values for this SNP*

```
  CCTGATCC GTTGAGATGC AGACAAGATG TATTTGCTAT GGATAGCAGA GCTGGAAAAC
K
ATCMCTGCCA AGCCCTGTGG AGCCTAGCAG ATCACTGAAA CCCCGCAGTC TCGTACTG

K= G/T
```

Note: Sequence in lower case indicates low-complexity or repetitive sequence

BLAST SNP flanking sequence against the mouse Genome

Assay ID	Submitter SNP ID	Submitter Handle	Population	ss orient	Variation Type	C57BL/6J	SPRET/EiJ
ss3175869	M-11A-B	MITCCR	Mouse (BJ)	f	SNP	G	T

SNP Consensus Information

SNP	Consensus Type	C57BL/6J	SPRET/EiJ
rs2228909	SNP	G	T

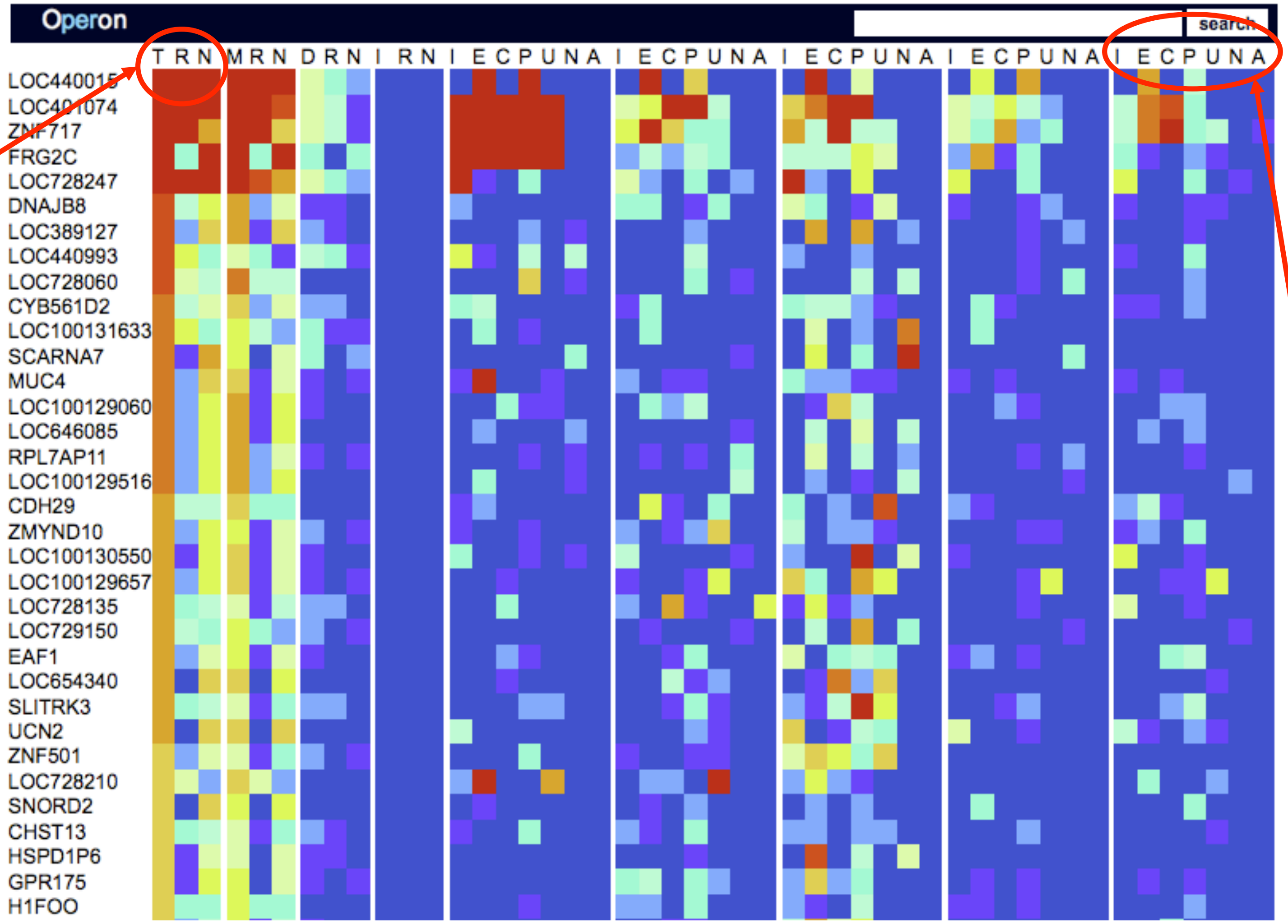
Sample order: ENU 01, ENU

	marker	position
1	rs2228909	35
2	rs3656412	41
3	sv55920684	43
4	sv55920685	47
5	sv55920686	47
6	sv55920687	
7	sv55920688	7131967
8	sv55920689	11787185

7	sv55920688	7131967	T	T	T	G	GT	4	0	0	4	19	TT	3	0	0	0	10
8	sv55920689	11787185	T	T	T	G	TT	14	0	0	0	45	GT	7	0	0	6	27

	G	T
1	27	0
2	0	38
3	0	0
4	7	0
5	0	0
6	0	0
7	0	10
8	6	27

Identification of gene variants



T=Total
R=Repeat
N=None repeat

Individual CHC433: I=Intron, E=End, C=Coding, P=Promoter, U=UTR, N=ncRNA, A=AAchange

Advantages of WG sequencing of ENU mutants

Traditional ENU screen

- Labor/time intensive intercrosses/backcrosses
- Careful choice of strain for intercross
- Needs sometimes large number (>100) animals for positional cloning
- Long, costly positional cloning (linkage study, candidate gene sequencing)

WG Sequence assisted ENU screen

- Can be carried out directly on G2 (dominant) or G3 (recessive) mice
- Can be carried out on same strain background
- Few animals are needed
- Directly provides candidate genes/mutations within weeks
- Facilitates identification of double hits

Conclusions/Outlook

- ENU mouse models can help understand gene function by providing phenotypes due to point mutations, even in cases where ko models are lethal.
- The combination of ENU mouse models models with new sequencing technology can accelerate the identification of the phenotype causing mutations from months/years to weeks.
- As most ENU mutations inducing phenotypic changes are exonic, exon capture prior to sequencing could further decrease complexity of the analysis.

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Thank You for Your Attention