B6 Albino *A*$^{++}$ Mutant Mice as Embryo Donors for Efficient Germline Transmission of B6 ES Cells

Taconic
Webinar 2014-05-14
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A decade of gene targeting in B6 ES cells at TaconicArtemis (2000 – 2010)

- ESC Manipulations:
  - 1840 B6 ESC transfections
  - 7826 clones individually frozen
- Embryo-Injections
  - 2133 Injection „Sessions“
  - 122184 blastocysts injected
- Mice work
  - 9869 B6 chimeras weaned
  - 3226 B6 chimeras mated

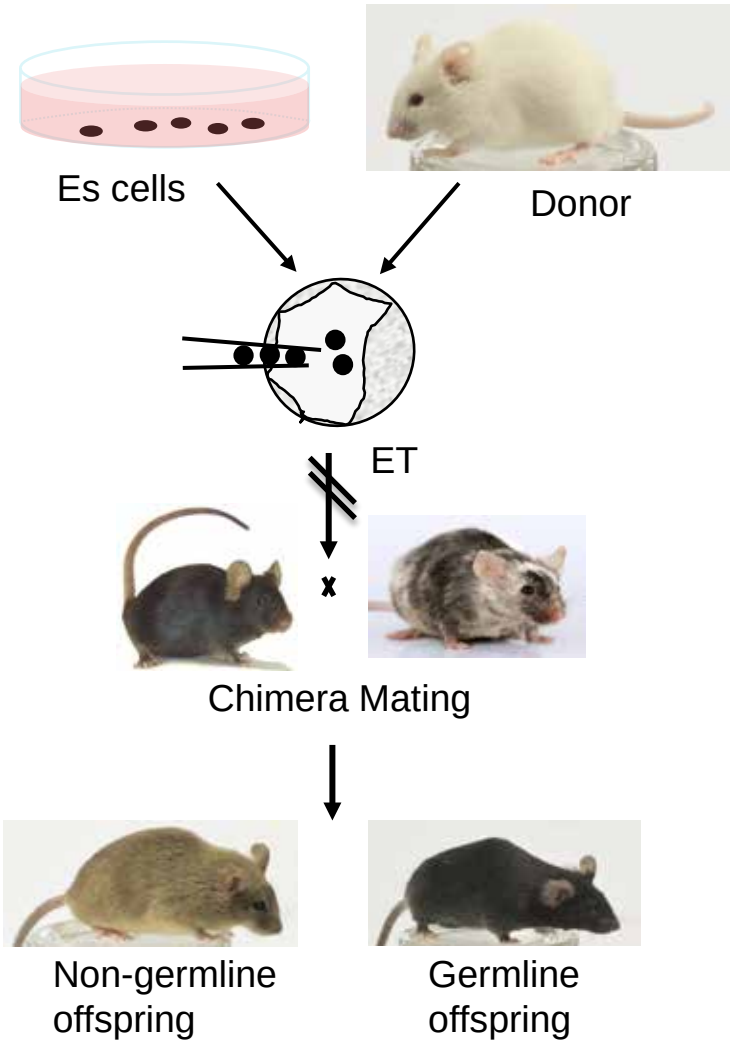
=> R&D on process optimization

Use of B6 ES cells has increased greatly

- Gertsenstein M. et al., (2010) PLoS One,.5(6):e11260)
  - **C2** ES cells derived from C57BL/6NTac
- EUCOMM
  - Uses primarily **JM8** ES cells, derived from C57BL/6NTac
- Knockout Mouse Project (KOMP)
  - JM8 and **VGB6** ES cells, derived from C57BL/6NTac,
  - **EAP1** ES cells, derived from C57BL/6N

=> Requirement for optimized protocols and tools for ES cell based transgenesis

Focus: Donor of embryos for injection of B6 ESC



- Should allow maximal contribution of ES cells to embryonic development
  - Preferrably ‚Inbred ES cell' <-> ‚Inbred host embryo' combination
- Should have a maximally different coat color compared to the ES cell background to allow judgement of ES cell colonization
  - "Black" 6 ES cells <-> albino donor strain
- Should allow detection of transmission of the ES cell genome by coat color in 100% of offspring
  - Example BALB/c:
    - B6CF1 ‚non-germline' = agouti
    - B6 derived ‚germline' = black

Limitations of commonly employed embryo donor strains for B6 ESC injection

- C57BL/6, B6D2F2
  - No detection of coat colour chimerism (black on black), genotyping of all potential chimeras and germline mating of offspring required

- BALB/c
  - Poor response on superovulation
    - 3 – 4 blastocysts are harvested from one BALB/c donor on average.
    - Approximately 16 donor BALB/c mice are required for the injection of 1 ES clone.
  - Unequal and delayed development of blastocysts

- B6 albino strains (C57BL/6-*Tyr*$^{c\text{-}Brd}$, B6(Cg)-*Tyr*$^{c\text{-}2J/J}$)
  - Detection of germline transmission via coat color requires either
    - breeding onto the desired B6 (sub)strain
      - No coat color distinction, genotypic analysis of all offspring,or
    - breeding onto the B6 albino host strain
      - Substrain background not maintained, carry-over of mutated *Tyr* allele

- **Conclusion**

  The current limitations on embryo donors are ineconomical and contradictory to Animal Welfare Aims (**R**eduction of animal numbers used in research!)

- **Goal**

  Development of a better female donor mouse/embryo host for C57BL/6 based ES cell transgenesis

Basic coat colour genetics

- The dominant agouti locus (*A*) mainly determines agouti pigmentation, the true wild-type coat color of mice, through the agouti signaling protein

- C57BL/6 strains harbour a recessive mutation of the agouti locus, the nonagouti (*a*) allele. An 14.7 kilobase pair retrotransposon in the first intron of the agouti gene abolishes transcription of Agouti mRNA.

- Albinism (white coat color) is caused by non-functional mutations of the tyrosinase (*tyr*) gene and epistatic over agouti.

Silvers W., The Coat Colors of Mice, Springer, 1979;
Adapted by: Mouse Genome Informatics, June 2003, Revised January 2008

Melanin synthesis

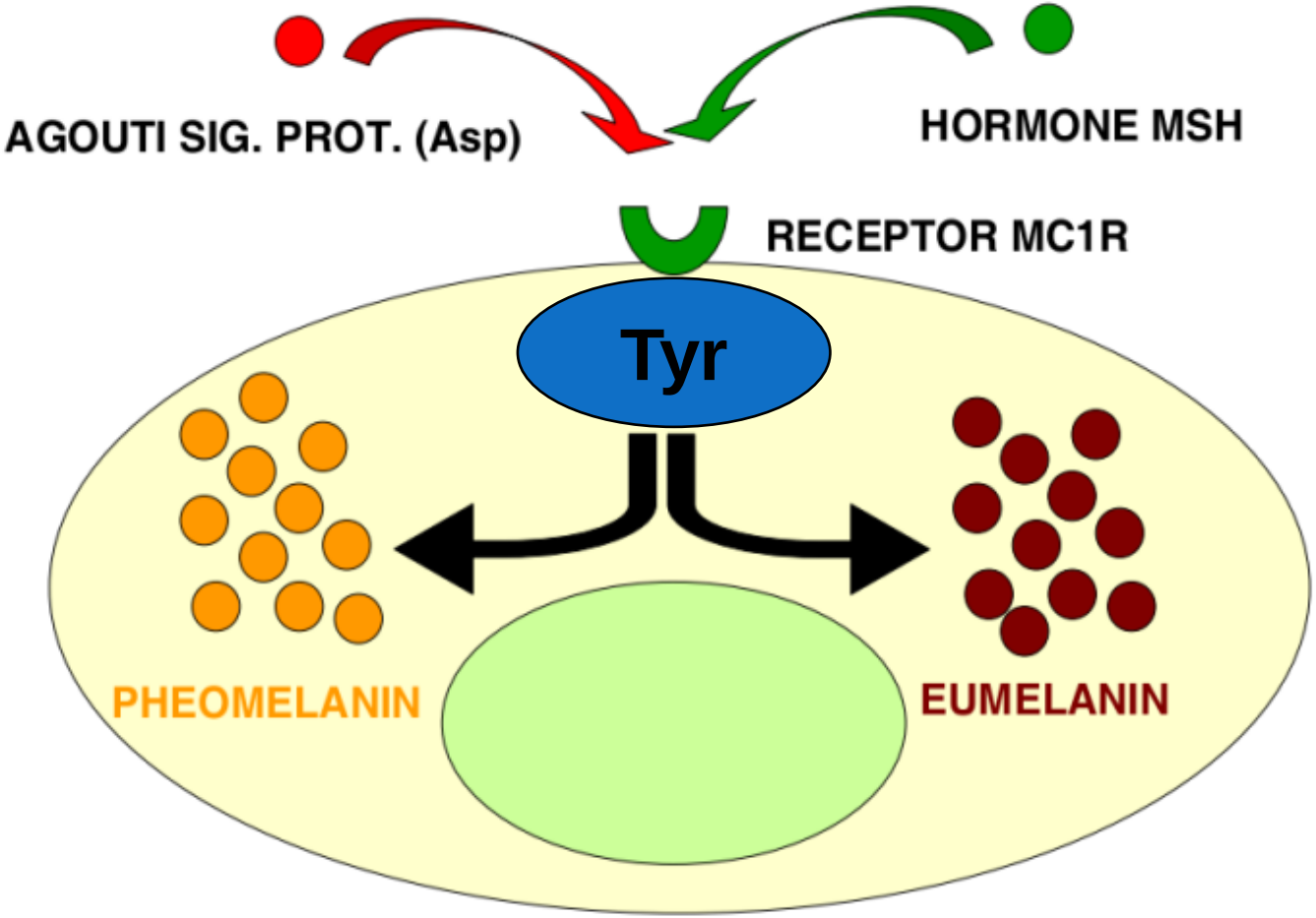


Illustration adapted from: Montoliu, L., TT2010 meeting, Berlin

Non-functional agouti signalling protein

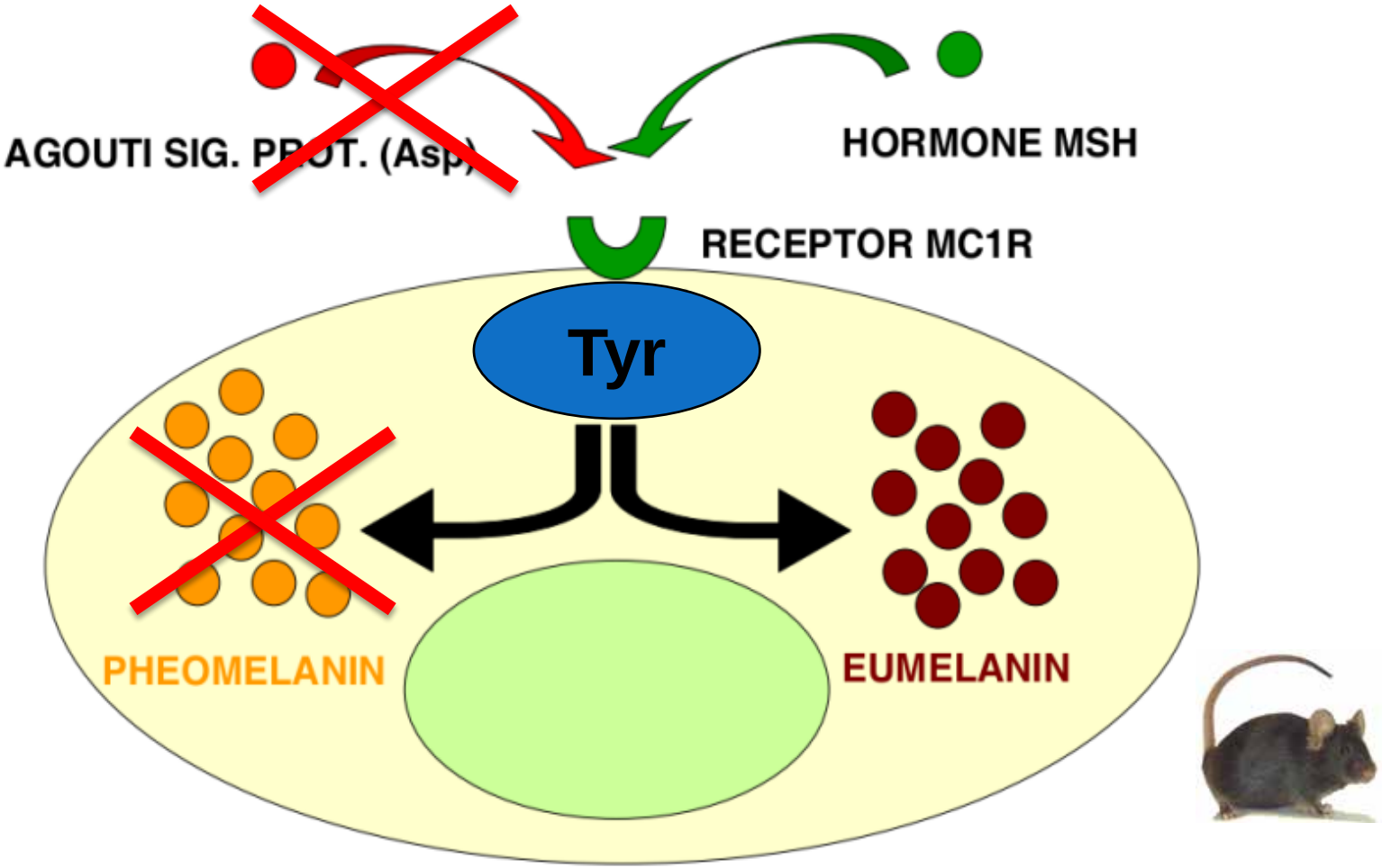


Illustration adapted from: Montoliu, L., TT2010 meeting, Berlin

Non-functional tyrosinase

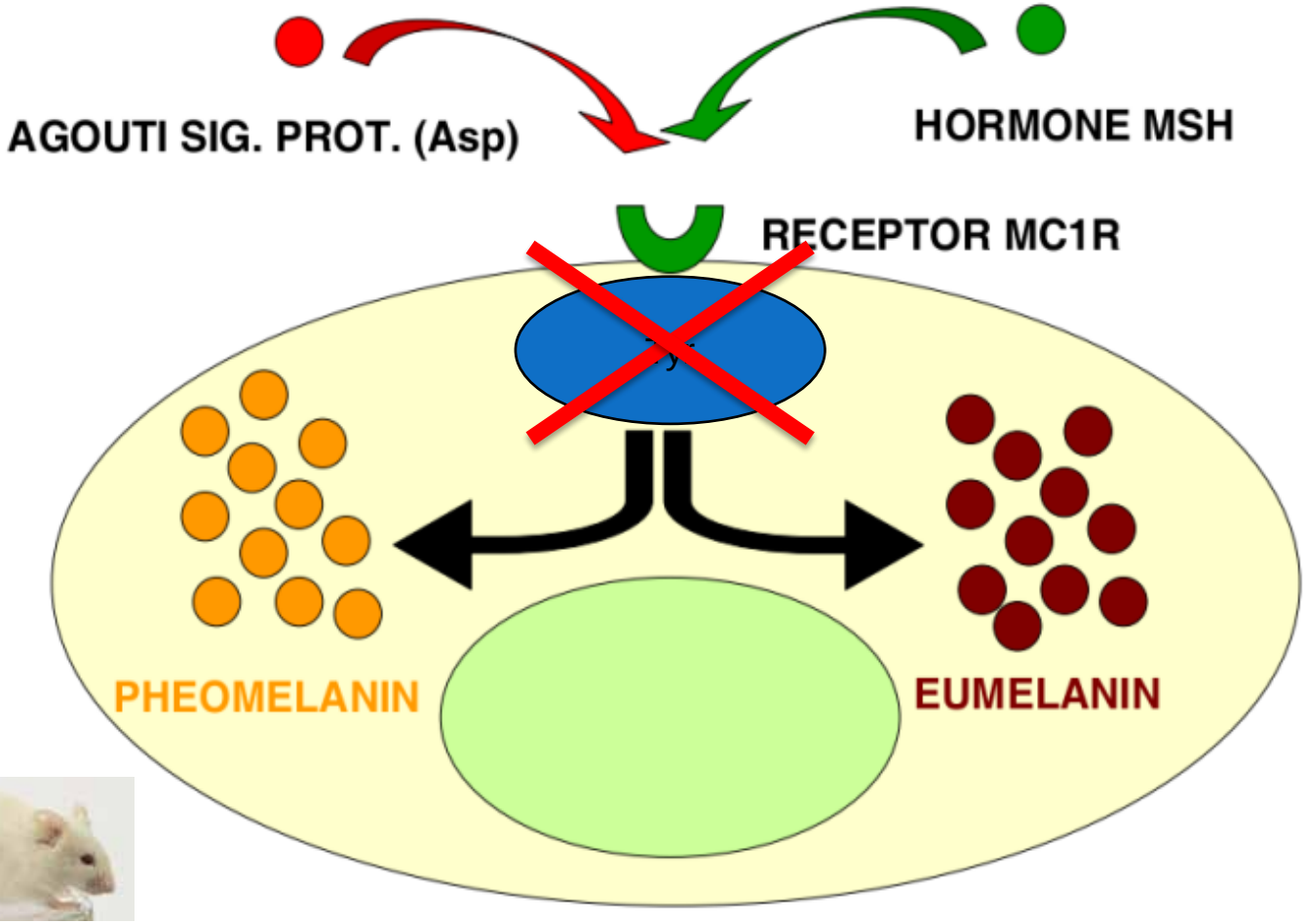


Illustration adapted from: Montoliu, L., TT2010 meeting, Berlin

Melanin synthesis is far more complex

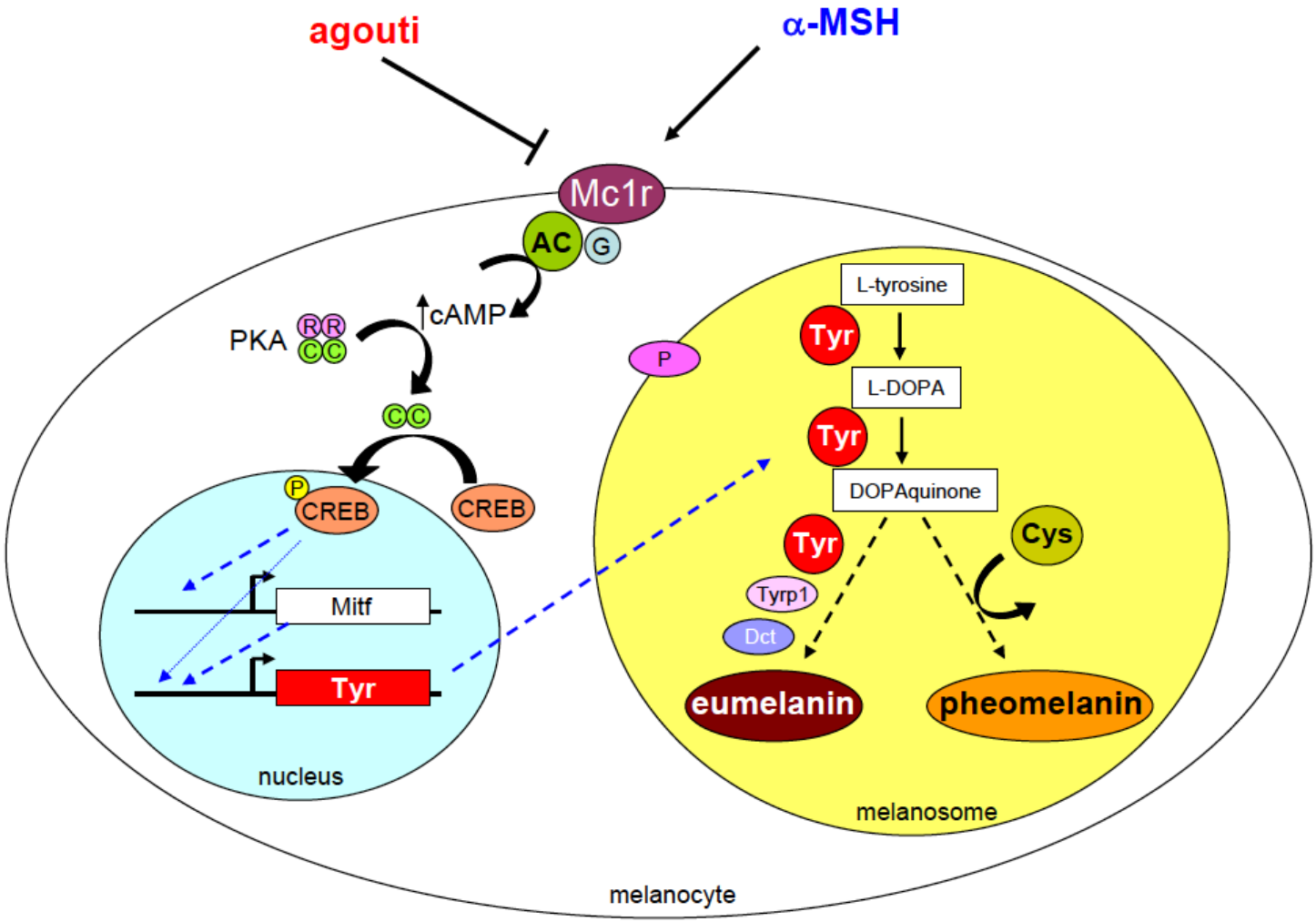
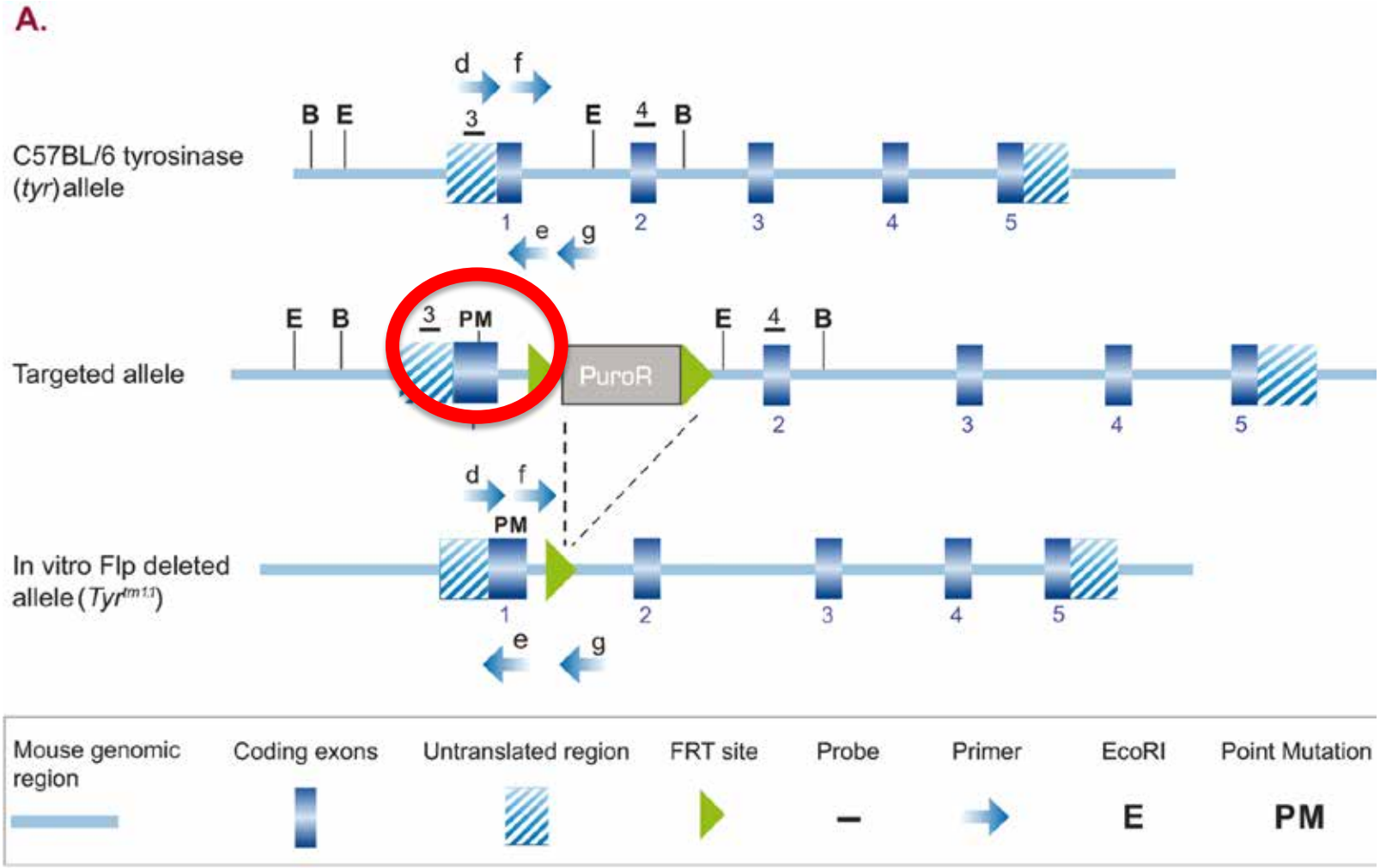


Illustration adapted from: Montoliu, L., TT2010 meeting, Berlin

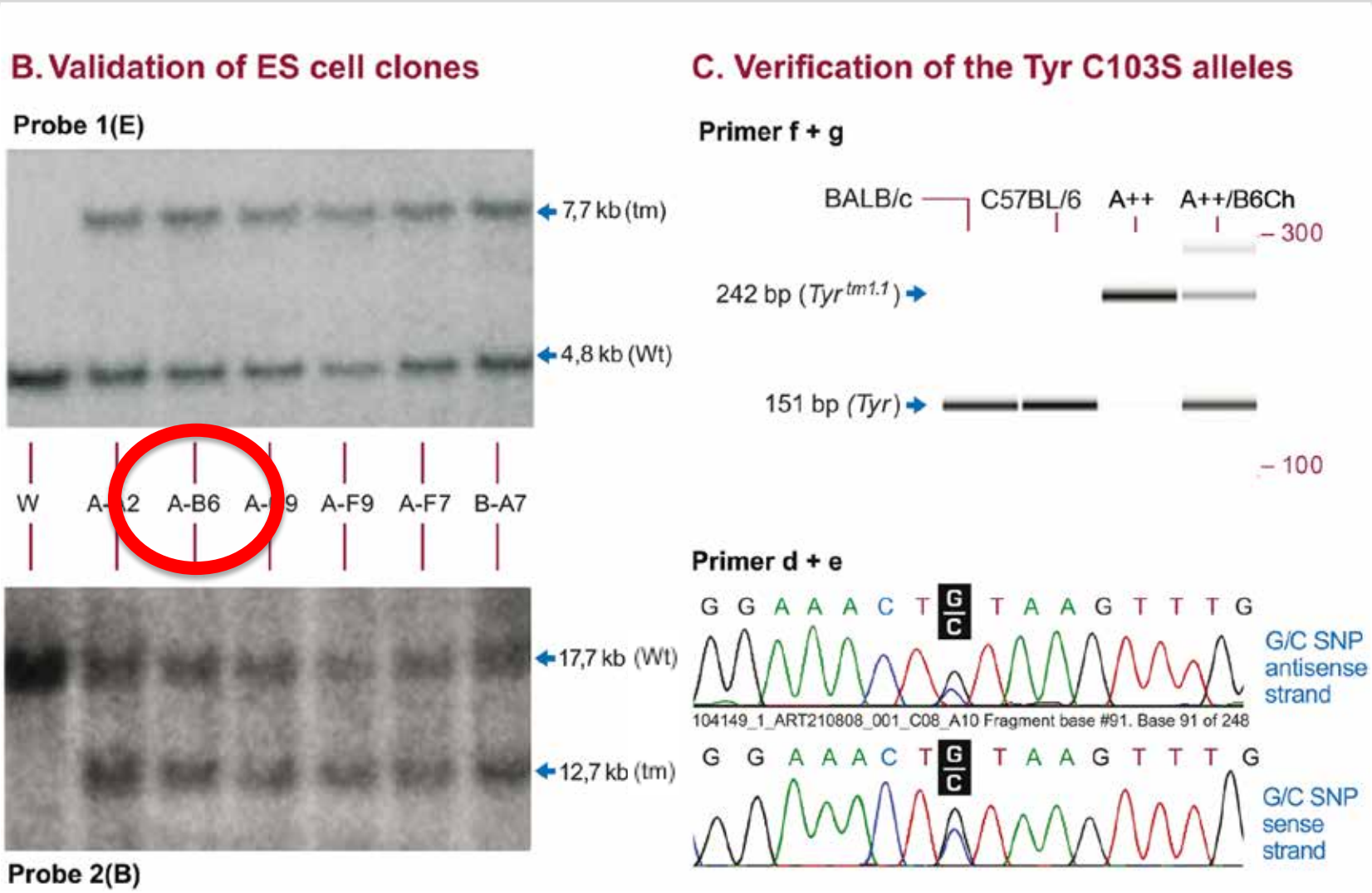
Generation of Albino and Albino *A*$^{++}$

1. **C57BL/6NTac-*Tyr*^{tm1Arte}**
   Gene targeting of the tyrosinase (*tyr*) gene:
   Introduction of a point mutation in exon 1 (C103S)

Inactivation of the tyrosinase locus
Gene targeting strategy



Inactivation of the tyrosinase locus
Validation

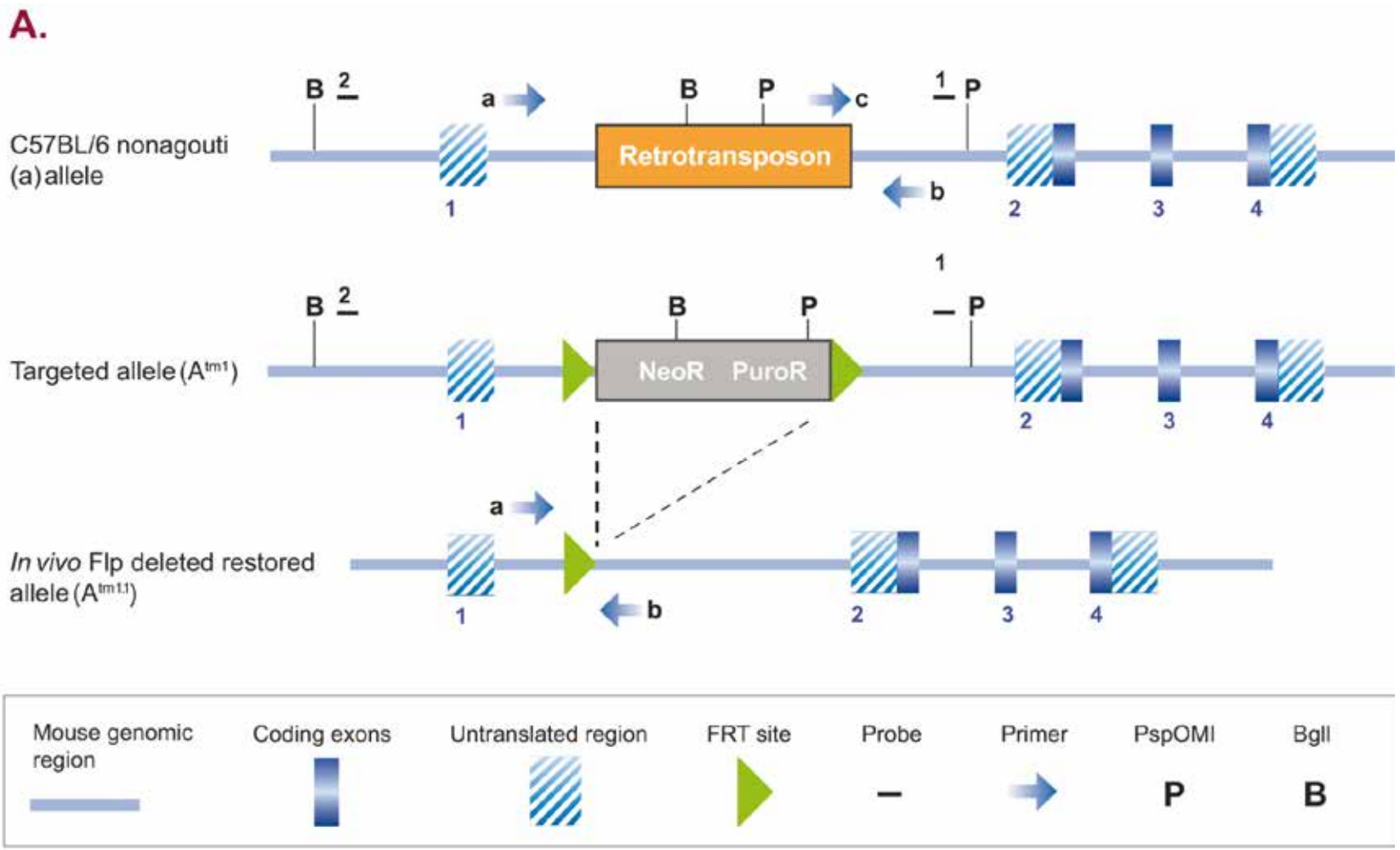


Generation of a compound B6 mutant host

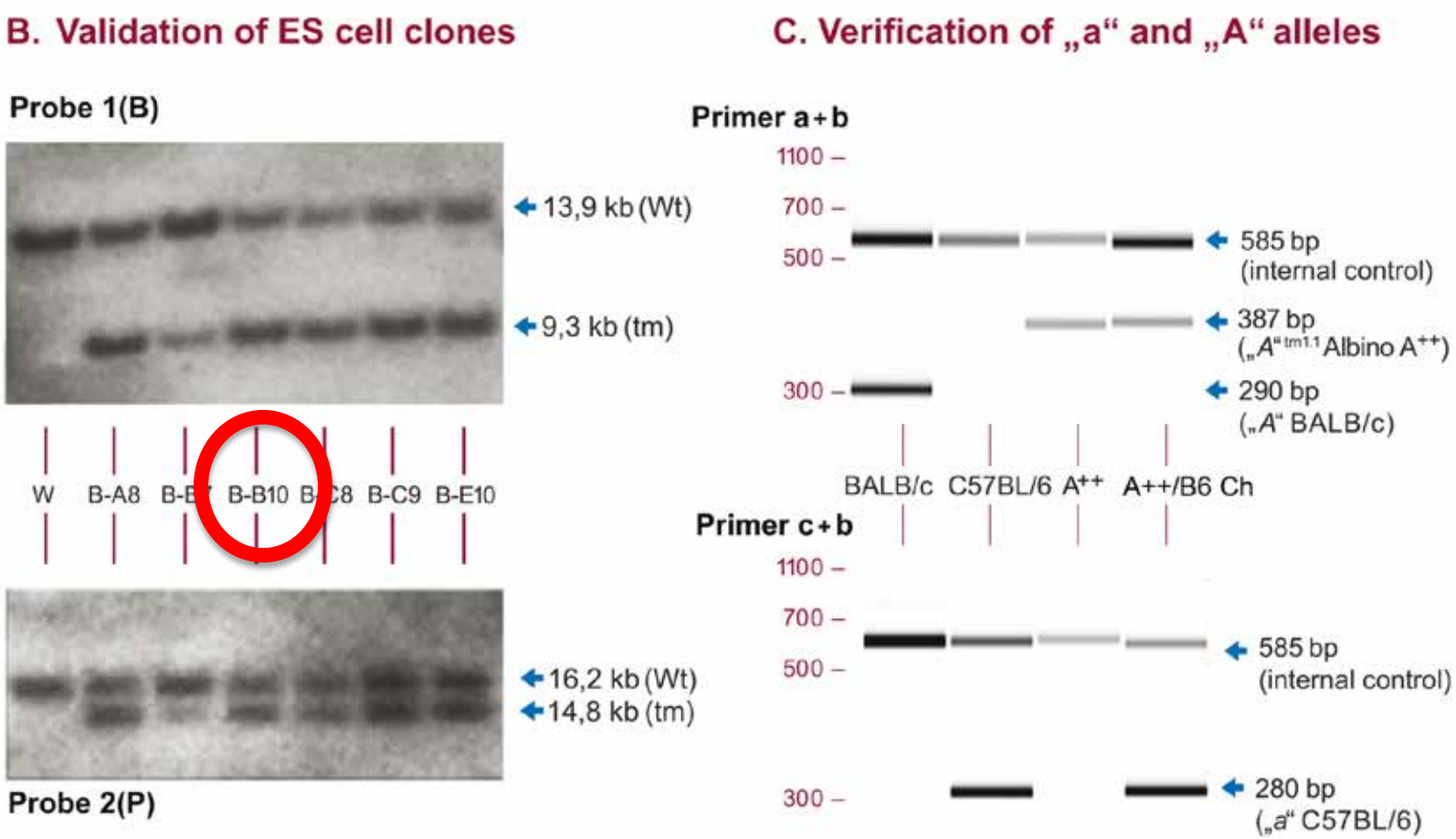
2. **C57BL/6NTac-*A*$^{tm1.1Arte}$**

   Targeted reversion of the non-agouti locus (a) to agouti (A).
   Deletion of the 14.7 kb retrotransposon.
   Phenotype in B6: agouti coat colour (A dominant over a)

Restoration of the agouti locus
Gene targeting strategy



Restoration of the agouti locus
Validation



Generation of a compound B6 mutant host

1. **C57BL/6NTac-*Tyr*^{tm1Arte}**
   Gene targeting of the tyrosinase (*tyr*) gene to create an albino B6 mouse

2. **C57BL/6NTac-*A*$^{tm1.1Arte}$**
   Targeted reversion of the non-agouti locus (a) to agouti (A).
   Phenotype in B6: agouti coat colour (A dominant over a)

3. **C57BL/6NTac-*A*$^{tm1.1Arte}$ *Tyr*^{tm1Arte}**
   Breeding homozygous double mutants. Phenotype albino (*Tyr* epistatic over A)

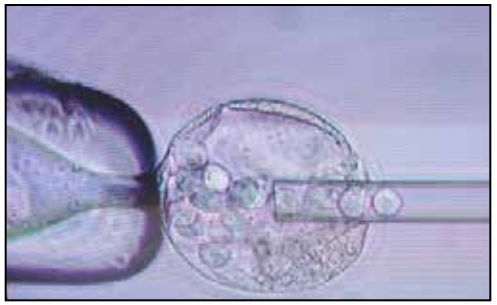
SNP confirmation

SNP	Chr	Position	C57BL/6NTac	albino A++	C57BL6/J
Mus-Ptprc	1	52665170	C	C	C
rs13476148	1	142911628	A	A	C
CEL-2_23847726	2	23847726	C	C	A
rs13476554	2	67180899	T	T	A
rs13477019	3	23589162	A	A	T
Mus-Tyrp1B	4	80481305	G	G	G
rs13477622	4	28491198	G	G	A
rs3662161	5	114905705	G	G	A
rs13478783	6	60682681	G	G	A
rs13478995	6	117862153	C	C	G
Mus-TyrC	7	94641553	G	C	G
rs13479522	7	116540646	G	G	A
rs13480100	9	21200544	G	G	A
rs13480122	9	31136193	G	G	A
rs13480619	10	57805922	G	G	A
rs29359333	10	57796761	T	T	C
rs13480759	10	109059096	A	A	G
rs13481014	11	47757117	G	G	A
rs13481573	12	82237479	A	A	G
rs13481634	12	101558810	C	C	A
rs13481734	13	26416832	G	G	A
CEL-14_116404928	14	116404928	A	A	G
rs4165065	16	17188907	G	G	A
rs13483055	17	58655424	G	G	A
rs13483237	18	19671420	G	G	C

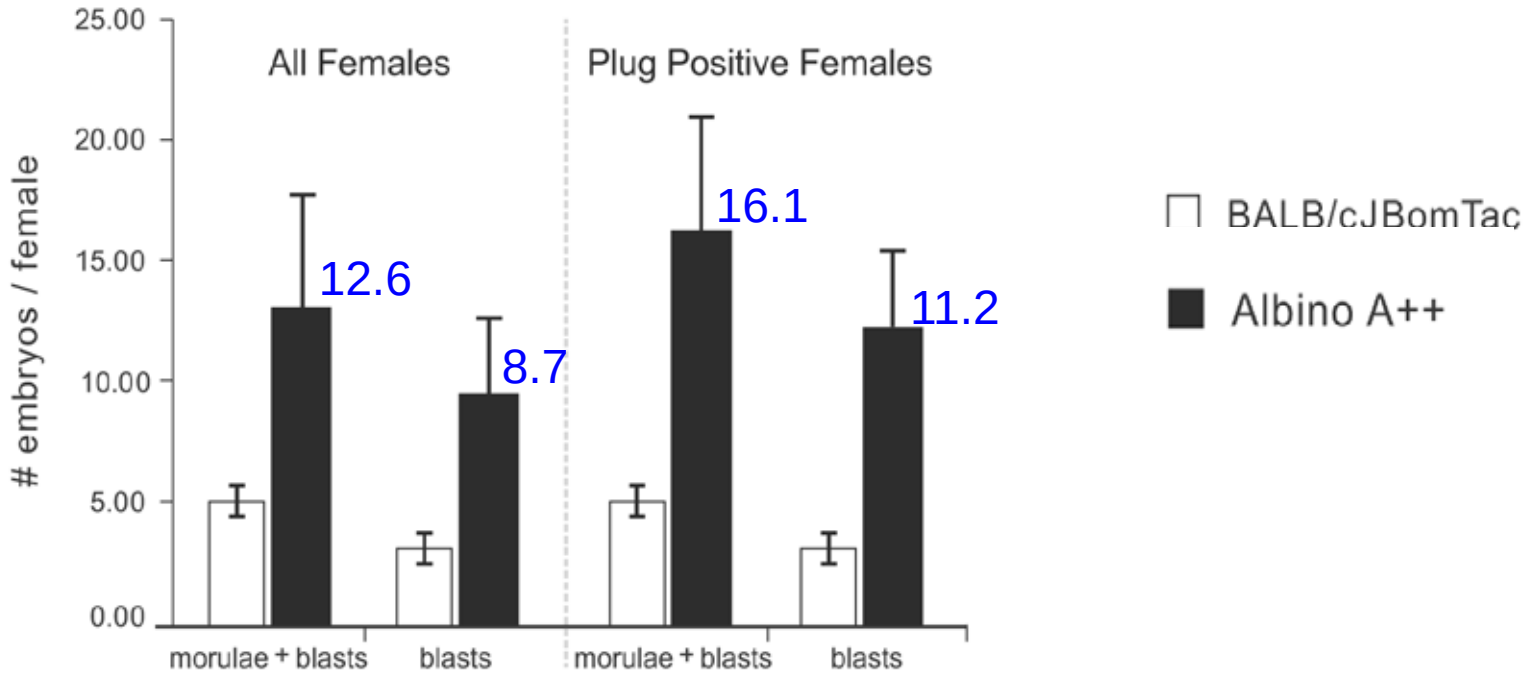
SNP confirmation of the C57BL/6NTac substrain identity of Albino A++ (blue), compared to C57BL/6J (red). The gene-targeted *Tyr* nucleotide exchange from G to C resulting in a C103S amino acid substitution in Albino A++ is highlighted.

Initial superovulation protocol

- B6 ESC diploid blastocyst injections:
  - BALB/c donor
    - On average 8 wks
  - Albino *A*++ donor
    - On average 4 – 5 wks
  - 5 IU PMSG <- 46h -> 5 IU HCG
  - Light cycle 12:12 (light:dark)
  - Limited expansion of harvested morulae at day of injection
  - Piezo-assisted injection of 15 ES cells/blc
  - Injection of 48 blc/ES clone
  - Bilateral transfer of 16 blc/NMRI foster



Superovulation response of Albino *A*$^{++}$



- Higher yield of embryos:
  - Superovulation response 2.5 – 3 x better compared to BALB/cJBomTac
- More equal embryonic development:
  - 70% harvested blastocysts at dpc 3.5, compared to 53% in BALB/cJBomTac

Chimeras and GLT of C57BL/6NTac targeted ES clones.

	Albino A++ (%)		Albino (%)	
# C57BL/6NTac ES Clones injected	21		3	
# pups born	210		20	
# chimeras born (% of live born)	156	74%	19	95%
# male chimeras weaned (% of chimeras born)	142	91%	12	63%
# > 50% chimeric males (% of chimeras born)	89	57%	9	47%
# clones test mated	6		2	
# chimeras tested	17		5	
# chimeras sterile	3		0	
# GLT* chimeras (% of chimeras mated)	14	82%	5	100%
# GLT clones (% of clones mated)	6	100%	2	100%

* GLT indicates germline transmission

1st Summary

	BALB/c	Albino	Albino *A*$^{++}$
+ C57BL/6 ES cell injection			
Superovulation response	poor	good	good[1]
Chimera coat color recognition	Yes (black/agouti/white)	Yes (black/white)	Yes (black/agouti/white)
+ C57BL/6 x chimera mating			
Coat color recognition of G1 GL offspring	Yes	No	Yes (black or agouti)
Maintenance of substrain specificity in G>0	No	Yes	Yes

1 = Ca. 2.5 x less donors needed to produce sufficient chimeras for germline transmission of ES cells

Use of Albino/-A++ host in various ES cell and mating partner combinations

I) Albino A++ host Strain: C57BL/6NTac-$A^{tm1.1Arte}Tyr^{tm1}$ (allele configuration: $A^{tm1.1}/A^{tm1.1}$, Tyr^{tm1}/Tyr^{tm1})

	ES cell	Chimera Coat Color	Mating Partner	Offspring Non-Germline	Offspring Germline	
1a	B6.3-6, JM8, C57BL/6NTac (a/a, Tyr/Tyr)	C57BL/6NTac + C57BL/6NTac ($A^{tm1.1}/A^{tm1.1}$, Tyr^{tm1}/Tyr^{tm1}) + (a/a, Tyr/Tyr)	C57BL/6NTac (a/a, Tyr/Tyr)	C57BL/6NTac ($A^{tm1.1}$/a, Tyr^{tm1}/Tyr)	C57BL/6NTac (a/a, Tyr/Tyr)	
1b	B6.3-6, JM8, C57BL/6NTac (a/a, Tyr/Tyr)	C57BL/6NTac + C57BL/6NTac ($A^{tm1.1}/A^{tm1.1}$, Tyr^{tm1}/Tyr^{tm1}) + (a/a, Tyr/Tyr)	C57BL/6NTac ($A^{tm1.1}/A^{tm1.1}$, Tyr^{tm1}/ Tyr^{tm1})	C57BL/6NTac ($A^{tm1.1}/A^{tm1.1}$, Tyr^{tm1}/Tyr^{tm1})	C57BL/6NTac (a/$A^{tm1.1}$, Tyr/Tyr^{tm1})	
2a	JM8A.3 (C57BL/6NTac) (A^{tm1Brd}/a, Tyr/Tyr)	C57BL/6NTac + C57BL/6NTac ($A^{tm1.1}/A^{tm1.1}$, Tyr^{tm1}/Tyr^{tm1}) + (A^{tm1Brd}/a, Tyr/Tyr)	C57BL/6NTac (a/a, Tyr/Tyr)	C57BL/6NTac ($A^{tm1.1}$/a, Tyr^{tm1}/Tyr)	C57BL/6NTac (a/a, Tyr/Tyr)	C57BL/6NTac (A^{tm1Brd}/a, Tyr/Tyr)
2b	JM8A.3 (C57BL/6NTac) (A^{tm1Brd}/a, Tyr/Tyr)	C57BL/6NTac + C57BL/6NTac ($A^{tm1.1}/A^{tm1.1}$, Tyr^{tm1}/Tyr^{tm1}) + (A^{tm1Brd}/a, Tyr/Tyr)	C57BL/6NTac ($A^{tm1.1}/A^{tm1.1}$, Tyr^{tm1}/ Tyr^{tm1})	C57BL/6NTac ($A^{tm1.1}/A^{tm1.1}$, Tyr^{tm1}/Tyr^{tm1})	C57BL/6NTac 50% (a/$A^{tm1.1}$, Tyr/Tyr^{tm1}) 50% ($A^{tm1Brd}/A^{tm1.1}$, Tyr/Tyr^{tm1})	

II) Albino host Strain: C57BL/6NTac-$Tyr^{tm1.1Arte}$ (allele configuration: a/a, Tyr^{tm1}/Tyr^{tm1})

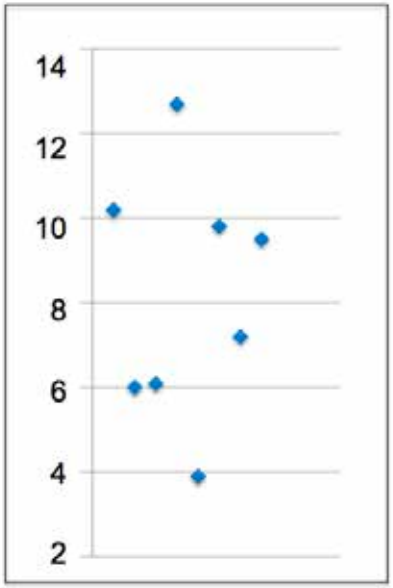
	ES cell	Chimera Coat Color	Mating Partner	Offspring Non-Germline	Offspring Germline	
1a	B6.3-6, JM8, C57BL/6NTac (a/a, Tyr/Tyr)		C57BL/6NTac (a/a, Tyr/Tyr)	C57BL/6NTac (a/a, Tyr^{tm1}/Tyr)	C57BL/6NTac (a/a, Tyr/Tyr)	
1b	B6.3-6, JM8, C57BL/6NTac (a/a, Tyr/Tyr)		C57BL/6NTac (a/a, Tyr^{tm1}/Tyr^{tm1})	C57BL/6NTac (a/a, Tyr^{tm1}/Tyr^{tm1})	C57BL/6NTac (a/a, Tyr/Tyr^{tm1})	
2a	JM8A.3 (C57BL/6NTac) (A^{tm1Brd}/a, Tyr/Tyr)	C57BL/6NTac + C57BL/6NTac (Tyr^{tm1}/Tyr^{tm1}) + (A^{tm1Brd}/a, Tyr/Tyr)	C57BL/6NTac (a/a, Tyr/Tyr)	C57BL/6NTac (a/a, Tyr^{tm1}/Tyr)	C57BL/6NTac (a/a, Tyr/Tyr)	C57BL/6NTac (A^{tm1Brd}/a, Tyr/Tyr)
2b	JM8A.3 (C57BL/6NTac) (A^{tm1Brd}/a, Tyr/Tyr)	C57BL/6NTac + C57BL/6NTac (Tyr^{tm1}/Tyr^{tm1}) + (A^{tm1Brd}/a, Tyr/Tyr)	C57BL/6NTac (a/a, Tyr^{tm1}/Tyr^{tm1})	C57BL/6NTac (a/a, Tyr^{tm1}/Tyr^{tm1})	C57BL/6NTac (a/a, Tyr/Tyr^{tm1})	C57BL/6NTac (A^{tm1Brd}/a, Tyr/Tyr^{tm1})

- Zevnik B. et al., (2014) C57BL/6N Albino/Agouti Mutant Mice as Embryo Donors for Efficient Germline Transmission of C57BL/6 ES Cells. PLoS ONE 9(3): e90570.

Need for optimized superovulation protocol

Observation

- Standard SOV protocol is only partly applicable for Albino A^{++}
  - Injectable embryo yield varies
    - Taconic experience:
      - on average **7.2** injectable embryos/donor employed
    - Independent test users (8 labs)
      - Range between 3.9 and 12.7 injectable embryos/donor employed
  - Embryos are too far advanced (hatching) at the time of injection
    - Difficult manipulation
    - Lower birth rates and lower chimerism
      - Diminished germline transmission rates



Optimized superovulation protocol

- 4 - 5 wk old female
- Light cycle 12:12 (light:dark)
- 5 IU PMSG at **3 pm** on day 1
- 5 IU HCG at **1 pm** on day 3
- Mate directly
- Collection at 3.5 dpc, start 6 am
- Incubate for **max. 1h at room temperature**
- Piezo-assisted injection of **7 – 9 ES cells/blc**
- Bilateral transfer of 16 blc/NMRI foster

Recommendation

- Shorten time from SOV to harvest by ca. 2 hrs
- inject fewer ES cells per blastocyst

Optimized protocol: Evaluation of chimera generation

Injection of gene-targeted C57BL/6NTac ES clones

	Albino *A*$^{++}$
# embryos transferred	1002
# live pups (% /transferred)	353 (35,2%)
# live chimeras	231 (65,4%)
Chimeras/blastocyst transf.	23,1%
> 50% chimeras (%/all ch.)	116 (50,2%)
# embryos injected/ch.	9
# donor mice to generate 1 chimera	1,2

Optimized protocol: Evaluation of chimera matings

Injection of gene-targeted C57BL/6NTac ES clones

	Albino *A*$^{++}$
# chimeras test mated	27
# GLT chimeras (%)	17 (63%)
No GLT	3 (11%)
No offspring	7 (26%)

High number of chimeras unable to sire litters

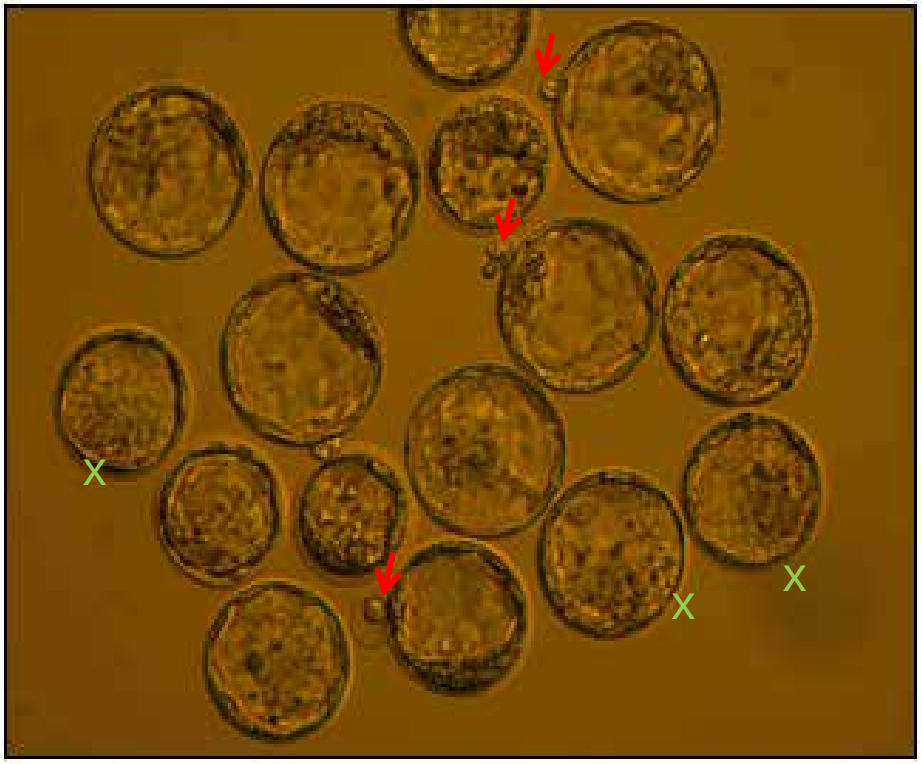
Comparison of chimera breedings

	Albino *A*$^{++}$	BALB/c
Average litter size	7,2 +/- 2,6	7,4 +/- 1,9
Average % germline pups	55,1% +/- 28,3%	65,5% +/- 36,1%
Average duration until first litter (d)	30,7 +/- 12,3	27,3 +/- 10,9

Differences between strains are not significant.

Practical hints I

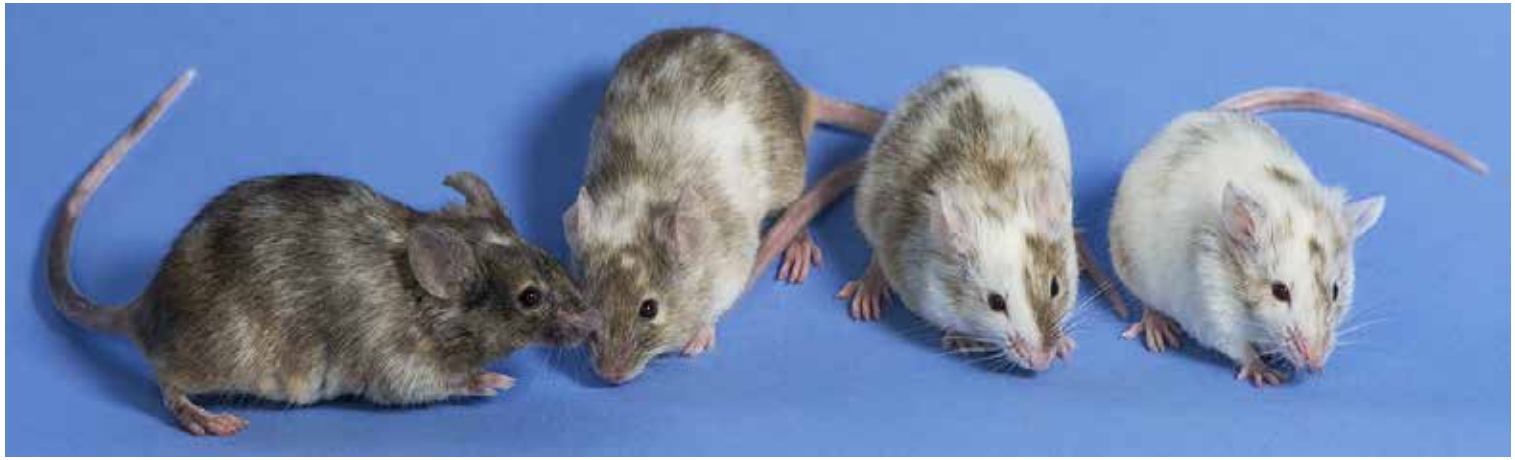
- Important to inject embryos in best phase (not under/over-developed)
  - chimerism is strongly dependent on this
- Do not inject too many ES cells; use 7-9 cells per embryo



Picture: NIH-funded research at UCONN-HARVARD and UCDavis

Practical hints II

- Chimeras generated from B6-derived ES cells and B6 Albino A^{++} blasts are tri-colored.
  - The agouti signalling peptide (produced in donor embryo-derived tyrosinase deficient cells), can produce a paracrine signalling effect in melanocytes derived from the ES cells. ES cell-derived hair patches can thus be either black or agouti. Both black and agouti patches should be counted for determination of chimerism percentages.



B6 ESC in B6-*Tyr*$^{c/c}$
Courtesy of S. Ortega, Spanish National Cancer Centre, Madrid, Spain

Summary

- C57BL/6NTac Albino or Albino A++ embryo donors display superior superovulation response compared to BALB/c
- Coat color recognition in chimeras is as predicted and equal to BALB/c hosts
- Upon injection with QC'd B6 ES cells, > 50% chimeras are generated at good frequencies (≥ 50%)
- Germline offspring is distinguishable by coat color as predicted
- Germline transmission frequency per chimera mated is equal or superior to standard published results (63 to 82%).
- Breeding performance of germline-transmitting chimeras equals standard results.
- C57BL/6NTac substrain specificity is maintained

Þ ***Conclusion***

Albino or Albino A++ are an optimized ‚tool' for ES cell based transgenesis, both in economic and animal welfare terms.