Overview

Embryonic Stem Cell Gene Targeting and Chimera Production in Mice

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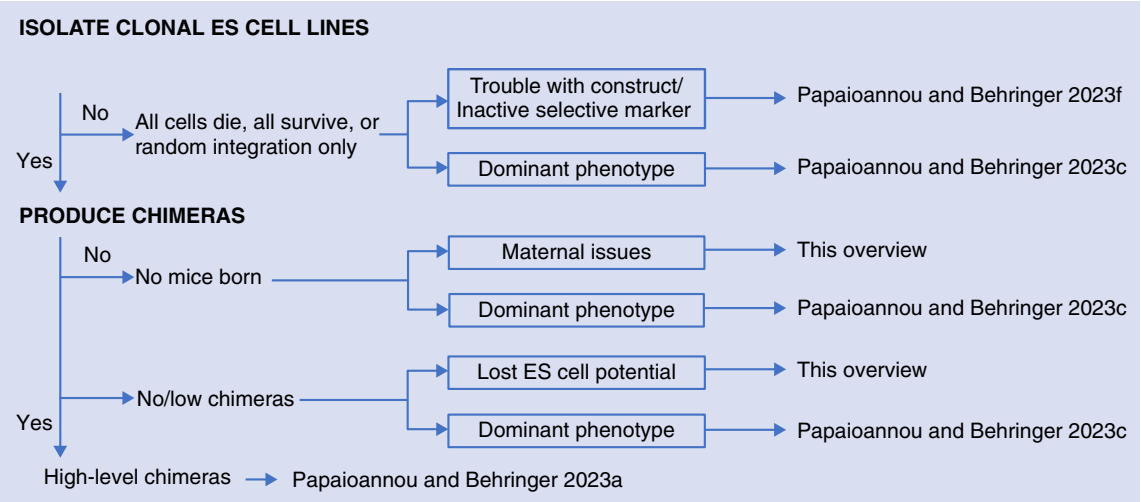
Producing a custom gene mutation in embryonic stem (ES) cells, whether through homologous recombination or CRISPR–Cas gene editing, is the first step along the way to getting the mutation into live mice. However, there are a number of additional steps along the way, each presenting technical challenges. Here, we provide a guide for troubleshooting when the results are not as expected and to distinguish technical problems from possible biological effects of the mutation. From the isolation of clonal lines of targeted ES cells through the production of ES cell chimeras with the targeted ES cell clone, we discuss common technical problems and their most likely causes and solutions. We also provide guidance for situations where the mutation has a phenotype in the form of a dominant effect on ES cells or in chimeras.

INTRODUCTION



The means to an end in science are often circuitous as unexpected results open new avenues of investigation. Sometimes these lead to serendipitous discoveries, but just as often they can be an annoyance and distraction if attainment of the intended end point is delayed. This overview is designed to guide you through the minefield of technical and biological issues that can sidetrack the recognition of

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From the Mouse Phenotypes collection by Virginia E. Papaioannou and Richard R. Behringer.

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BOX 1. TEN MOST COMMON MISTAKES IN ES CELL GENE-TARGETING EXPERIMENTS AND THEIR CONSEQUENCES

1. Starting with 'bad' ES cells, that is, cells that are not germline competent.
2. Forgetting to add targeting vector DNA to the cuvette before electroporation; all ES cells die during selection.
3. Using the wrong G418 concentration; no cells die or all cells die.
4. Using β-mercaptoethanol at too high a concentration; all cells die.
5. Electroporating ES cells that were already drug-resistant; all cells live.
6. Switching the lids of the 96-well plates during the freeze down of the master plate or inverting the orientation of the duplicate 96-well plate used for genotyping, leading to the use of the wrong ES cell clones to generate chimeras.
7. Killing ES cells during freeze down of the master plate; targeted clones can be identified but not retrieved.
8. Using the wrong probe to identify homologous recombinants; no targeted ES cell clones can be identified or nontargeted clones were used to make chimeras.
9. Retaining herpes simplex virus thymidine kinase in ES cells, causing male chimera sterility; no germline transmission of the ES-derived genotypes (see Box 3 in Overview: **Mouse Gene-Targeting Strategies for Maximum Ease and Versatility** [Papaioannou and Behringer 2023f]).
10. *loxP* site in the targeting vector is not functional; not discovered until after germline transmission and crosses with Cre mice.

a mutant phenotype in a gene-targeting experiment using embryonic stem (ES) cells in mice, whether you have produced the ES cell mutation by homologous recombination or by gene editing using CRISPR–Cas in ES cells. If you are using CRISPR–Cas to target genes directly in embryos, you can skip this overview and go to Overview: **Recovering a Targeted Mutation in Mice from Embryonic Stem Cell Chimeras or CRISPR–Cas Founders** (Papaioannou and Behringer 2023a).

Once a targeting vector has been electroporated into ES cells, the next steps are to isolate clonal lines of correctly targeted ES cells and grow up a sufficient number for further experiments and future use, and then to make chimeras with the mutant cells. We assume that you started your gene-targeting experiment with a source of proven germline competent ES cells and that you are following the detailed protocols provided by other sources for their culture, electroporation, and selection (see Chapter 11 in Behringer et al. 2014 and Joyner 1993). In this overview, we address some of the pitfalls that might be encountered in recovering clonal lines of correctly targeted ES cells and making germline chimeras, including the possibility that you might have early indications of a dominant phenotypic effect, and direct you to the next steps in diagnosing problems or moving ahead with the analysis. Ruling out the common mistakes in gene-targeting experiments will lead to the most efficient way of determining the effects of a mutation and steers you clear of time-consuming technical discoveries that others have made many times before you (Box 1).

ISOLATE CLONAL LINES OF CORRECTLY TARGETED ES CELLS

Careful design of a targeting construct and meticulous technique for ES cell culture, electroporation, and selection should lead to the successful isolation of correctly targeted ES cells that are easily propagated and expanded, allowing you to freeze aliquots as backup and for future use (see Chapter 11 in Behringer et al. 2014). If this is the case, you can skip this section and get on with making chimeras (Produce ES Cell Chimeras with Your Targeted ES Cell Clone, below). If not, there are a number of possible causes presented here in approximate order of decreasing likelihood that you will need to explore.

Technical Difficulties

If no or very few ES cell colonies survived drug selection, it is worth considering that you may have neglected to add the DNA targeting construct to the cell suspension before electroporation, failed to linearize it, or used the incorrect concentration, thus leaving all cells susceptible to the selective drug. Alternatively, you might have added too high a concentration of the selective drug. If you are using drug-resistant feeder cells, whether or not they die can provide an indication of whether the drug dose is toxic.

Other technical problems that might lead to the death of all cells, such as problems with the electroporator, culture medium, incubators, etc., can easily be ruled out by the simple control of culturing two extra dishes of ES cells—one electroporated, the other not—without the selective drug. These ES cells should grow well, whereas the experimental drug-treated cells should all die, apart from resistant colonies. Try another electroporation with this control, making sure that you add the DNA construct, to determine whether your electroporation and culture conditions are optimal.

If a large number of clones or a solid lawn of ES cells survives drug selection, but few or none have incorporated the targeting construct, you may have neglected to add the selective drug or used an inadequate dose of the drug. Or perhaps you thawed the wrong vial of ES cells for the electroporation and used some that were already drug-resistant. It is always worth determining a "kill curve" for a particular ES cell line and batch of drug so that you know what to expect. Simply determine a dose–response curve for the drug, selecting the lowest dose that kills 100% of exponentially growing cells within 5–7 d.

Trouble with the Targeting Construct

If you are getting drug-resistant colonies with random integrants of your targeting construct, but no homologous recombination at the targeted locus, it could be that you have a less-than-perfect targeting construct or that your locus is hard to target. Before going further, make sure that you are using the correct genotyping probe. Is the band of the correct, predicted size? It is worthwhile to sequence the probe fragment to ensure that it is correct. If you are using polymerase chain reaction (PCR), double-check your strategy. Some loci are notoriously difficult to target but we have yet to hear a convincing explanation for this, or more importantly, how to predict it. Your best bet is to review Overview: **Obtaining or Generating Gene Mutations in Mice** (Papaioannou and Behringer 2023b) and make sure that you have done everything you can to optimize the chances of homologous recombination, particularly with respect to the use of isogenic DNA and to having sufficiently long arms of homology. If you have any doubt, consider redesigning and rebuilding the targeting construct. However, before you do, electroporate again with the construct that you have and screen for additional clones, which usually takes less time and effort than rebuilding the targeting construct. On the other hand, the low efficiency may be part of a learning curve that will improve with practice, so do not get discouraged.

Inactive Selectable Marker

If ES cells die, but you have ruled out the technical difficulties listed above and drug-resistant feeders are fine, a fairly remote possibility is that the selectable marker in the construct may be inactive, either because of mutations introduced during cloning or the activity of a transcriptional repressor in the region of homology used for building the construct. In the first case, you could sequence the selectable marker you used and replace it with another if it has been mutated. If this is not the problem, consider a different placement of the selectable marker in the construct—for example, use a different region of homology in hopes of avoiding the putative repressor.

Is This a Phenotype?

Finally, there is the possibility that *this is your phenotype!* Perhaps the mutation of a single copy of your favorite gene is sufficient to render the heterozygous ES cells incapable of growing—a dominant effect. Explore this last possibility but continue to work, because we know of no actual case of a gene with this

particular heterozygous mutant effect of killing ES cells. But how would you go about testing it if you think that you have that rare case? Go to Overview: **Phenotypic Analysis of Dominant Mutant Effects in Mice** (Papaioannou and Behringer 2023c) to investigate possible dominant effects. If you can rule out this possibility, try electroporating again and select a large number of clones for further analysis. You simply may not have tested enough clones to recover a rare homologous recombination event. If all else fails, make a new targeting vector incorporating greater or different regions of homology.

It should be noted that X-linked or Y-linked genes are special cases in that only one copy is present in XY ES cells, so the cells are functionally hemizygous for the gene except for genes located within the pseudoautosomal region shared by the X and Y chromosomes (Perry et al. 2001). The possibility that a mutation in the single copy results in an ES cell phenotype is thus more likely since it does not require a dominant effect.

EXPAND AND FREEZE CORRECTLY TARGETED ES CELL CLONES FOR FUTURE USE

How Many Clones Are Enough?

To verify that a phenotype is the result of the targeted mutation you created, it is useful to show that two independently targeted ES cell clones give the same phenotype. However, to be on the safe side, it is worth recovering more than two targeted clones—five to 10 would be reasonable. These cells have been subjected to electroporation, drug treatment, and cloning. Even if you started with a parent ES cell line with excellent germline potential (which you should have tested), the resultant clones could have picked up mutations, chromosomal aberrations, or restrictions of developmental potential during these procedures, so that any single clone might not provide germline transmission through chimeras. Similarly, if you have only one or two clones, they might succumb to some technical glitches along the way, so it is wise to have a few more as backup.

In the event that your targeting efficiency is very low and it is hard to get a reasonable number of targeted clones without massive effort, check the technical aspects of the electroporation procedures because this is often where the problem lies. Check the DNA concentration and purity. Make sure that the DNA is completely dissolved and well mixed with the ES cell suspension before electroporation. In addition, make sure to use the correct cell concentration and electroporation parameters. If the efficiency is still low and you have consulted Overview: **Mouse Gene-Targeting Strategies for Maximum Ease and Versatility** (Papaioannou and Behringer 2023d) for hints on optimizing the targeting construct, persevere a bit longer. In our experience, a definite learning curve exists and gene-targeting efficiency often improves with electroporation practice.

How Many Cells Are Enough?

As soon as you have identified a targeted clone, culture and expand it, verify by regenotyping that it is indeed targeted, and freeze down a sufficient number of cells for future experiments. Five to 10 vials of each targeted clone should be fine, but you might want to archive these in more than one location to guard against losing them all in a freezer disaster.

PRODUCE ES CELL CHIMERAS WITH YOUR TARGETED ES CELL CLONE

The next step in gene targeting using ES cells, whether you used homologous recombination or CRISPR–Cas gene editing, is to get the mutation into live mice through the production of chimeras that will pass the mutation through their germline. Generating mouse chimeras with correctly targeted ES cells can be done either by injection into blastocysts or by aggregation with or injection into morulae (see Chapter 12 in Behringer et al. 2014). Ideally, the ES cells will make a large contribution to the chimera that will be detectable by a high contribution to the coat color phenotype (i.e., high-level chimeras), provided you made use of a suitable coat color marker in your chimeras. If this is the case

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and you have obtained high-level chimeras with your targeted ES cells, you can skip the rest of this overview and go on to Overview: **Recovering a Targeted Mutation in Mice from Embryonic Stem Cell Chimeras or CRISPR–Cas Founders** (Papaioannou and Behringer 2023a). Here, we provide some pointers in planning the composition of your chimeras to take advantage of coat color markers, and in subsequent sections we troubleshoot possible reasons for a failure to obtain high-level chimeras.

Planning the Composition of Chimeras

The particular strain and substrain of origin of the ES cells you are working with will determine the most suitable host embryo strain to use in making chimeras. The best options will incorporate a coat color genotype that will allow visual identification of the contribution of ES cells to the resulting chimeras so that high-level chimeras can be easily identified and selected for subsequent recovery of the targeted mutation. However, you will also need to consider how you are going to breed the chimeras once they are identified for the next step of recovery of the mutation (see Breeding Schemes for ES Cell Chimeras in Overview: **Recovering a Targeted Mutation in Mice from Embryonic Stem Cell Chimeras or CRISPR–Cas Founders** [Papaioannou and Behringer 2023a]). Another practical consideration is the cost and ease of obtaining mice to provide the host embryos, so a readily available commercial strain is best. Remember that this strain is simply a temporary host that will deliver the targeted mutation into live mice, without making a permanent genetic contribution to the resulting mutant strain.

Historically, ES cells derived from 129 strain mice were the most commonly used for gene targeting because of the ease of obtaining the ES cells from embryos of this strain (Robertson 1987) and because of their propensity to colonize the germline in chimeric hosts. In terms of genetic purity, however, the 129 strain could hardly be a worse choice as there are a number of different substrains with significant genetic differences. Nonetheless, if your starting point in gene targeting was a 129-derived ES cell line, it is important to know what substrain you are working with. The 129 strain originated in 1928 from crosses of several fancy coat color mice. From these crosses, a variety of 129 substrains were developed either intentionally or simply by random genetic drift following separation of breeding colonies, and various contaminating crosses were made either intentionally or inadvertently. The result today is a range of 129 substrains differing or segregating for a variety of alleles, including coat color genes (Table 1). All of these, however, carry the dominant *white bellied agouti* allele (A^w), which simplifies the choice of embryo host for making chimeras as the chimeras made with any *non agouti* (*a/a*) strain, such as C57BL/6J or C57BL/6N, will be easily identified by agouti coat color patches (Fig. 1A).

In recent years, technical advances have made it possible to obtain germline-competent ES cells from many different strains, any of which might be used for gene targeting (van der Weyden et al. 2011). Of particular note are ES cells derived from the C57BL/6N substrain, which have been used in the high-throughput gene-targeting projects of the International Knockout Mouse Consortium (IKMC; https://www.mousephenotype.org/about-impc/about-ikmc/). If C57BL/6N-derived ES cells were used for gene targeting, there are a number of options for host blastocysts depending

TABLE 1. Derivation of some common 129-embryonic stem (ES) cell lines with the old and new 129 substrain nomenclature[a] and the relevant coat color alleles these ES cell lines carry

	Substrain of embryo of origin		
ES cell line	Old nomenclature	New nomenclature	Relevant coat color alleles in ES cells
AB1	129/SvEvBrd	129S5	A^w
CCE	129/SvEv	129S6	A^w
E14TG2a[b]	129/OlaHsd	129P2	A^w; $Tyr^{c\text{-}ch}$; p
J1	129/SvJae	129S4	A^w
R1	129/Sv × 129/J	129S1 × 129X1	A^w; $Tyr^{c\text{-}ch}$; Tyr^+; p; p^+

[a]http://www.informatics.jax.org/mgihome/nomen/strain_129.shtml
[b]The ES cell–derived component of chimeras made with this cell line will be very pale colored.

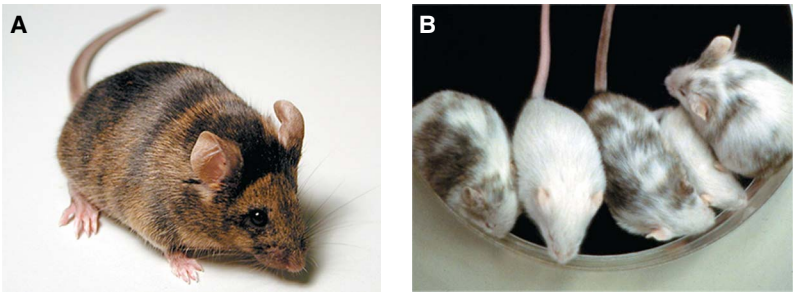

FIGURE 1. Examples of chimera coat colors. (*A*) The case of a 129-derived embryonic stem (ES) cell line combined with C57BL/6J (B6) blastocysts. The chimera has patches of agouti and black hairs by virtue of the *white-bellied agouti* (A^W/A^W) genotype of the ES cells and the *nonagouti* genotype (*a*/*a*) of the B6 embryos. This animal is somewhat >50% agouti and would be a reasonable candidate for test breeding. (*B*) Chimeras made with 129-ES cells and albino, non-*agouti* blastocysts (Tyr^c/Tyr^c; *a*/*a*) have white, black, and agouti patches. The white patches are areas populated by blastocyst-derived albino melanocytes, the agouti patches have 129-derived melanocytes and hair follicles (the site of action of the *agouti* gene), and the black patches have 129-derived melanocytes but blastocyst-derived (*a*/*a*) follicles. These animals range from ~5% to 50% chimerism.

on whether the ES cells have recessive *agouti* alleles (*a*/*a*, e.g., ES cell line JM8) or whether they contain a dominant *agouti* allele (*A*/*a*; e.g., ES cell line JM8A3 or JM8A1.N3) (Pettitt et al. 2009). Either will show up as pigmented patches if an albino host is chosen (Fig. 1B). To take advantage of the tried and true (and cost-effective) C57BL/6J strain as a host embryo, the chimeras will be evident by agouti patches only if the ES cell line carries a dominant *agouti* allele. Whatever strain is chosen for the host embryo, be sure to plan ahead and anticipate how you will recognize not just the chimeras, but also how you will test breed the chimeras for detection of germline transmission (see What Strain to Breed Chimeras with? in Overview: **Recovering a Targeted Mutation in Mice from Embryonic Stem Cell Chimeras or CRISPR–Cas Founders** [Papaioannou and Behringer 2023a]).

No Mice Born Following Transfer of Chimeric Embryos

Technique, Technique

Practice control embryo transfers until you have a good success rate (see Chapter 6 in Behringer et al. 2014). You should be able to achieve an 80%–90% success rate in embryo recovery after embryo transfer before you start with important experimental embryos. Otherwise, you are wasting your time. Try to reduce surgical trauma and, in particular, treat the uterus gently, because rough treatment can cause a pregnancy to fail. Make sure your embryo culture medium is correct (see Chapter 4 in Behringer et al. 2014). In addition, be sure that your pseudopregnant embryo transfer recipients are really pseudopregnant (male mice have been known to plug females that are not in estrus) by checking that they have corpora lutea (CL) in the ovary at the time that you do the embryo transfer (Protocol: **Counting Murine Corpora Lutea to Determine the Number of Oocytes Ovulated** [Papaioannou and Behringer 2023e]). If not, you are essentially throwing the embryos away, so use another recipient. Finally, check the light cycle control in your animal room to ensure that it is turning on and off correctly (14 h light/10 h dark is good, but 12/12 is fine, too), as erratic lighting disturbs the reproductive cycle.

Maternal Issues

It is possible, especially if you saw that the recipients were pregnant (i.e., they looked bulgy around the middle 10–12 d after embryo transfer), that pups were born but cannibalized before you had the chance to see them. Be aware of the date that the chimeras are due to be born and search the bedding material for telltale remains on that date (if you find any, even small remains can be genotyped to determine whether the newborns were chimeric). Sacrifice the surrogate female and examine the

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uterus shortly after the expected due date for signs that she recently gave birth. There will be red parturition sites on the mesometrial side of the flaccid, distended uterus if she gave birth.

If cannibalism seems to be the problem, there are several ways to improve this inadequate maternal behavior.

1. Minimize crowding during late pregnancy. Keep only one or two pregnant mice in the same cage (however, note that local institutional rules may dictate the number of animals per cage).
2. Avoid bedding or cage changes within 1–2 d of the expected birth date, but be sure that the cage is relatively clean. This means that you may have to put a "do not disturb" sign on the cage and take the responsibility of keeping it clean, fed, and watered rather than relying on the schedule of animal husbandry staff. Provide nesting material a few days in advance of the due date. You can use commercial nesting materials or you can place a tissue or paper towel in the cage.
3. No matter how anxious you are to see the babies, do not disturb the mother during or shortly after birth. If you observe the pups strewn around the cage and/or mutilated, then and only then is the time for drastic intervention in the form of cross-fostering onto a lactating female (Protocol: **Cross-Fostering of Newborn Mice to Counteract Bad Parenting** [Papaioannou and Behringer 2023f]). If cannibalization is a persistent problem, a last resort is to preempt the birth by delivering the pups by Caesarean section (see Chapter 6 in Behringer et al. 2014) a day before the expected birth date (E18.5) and cross-fostering onto a lactating female.

Phenotype

It is just possible that the mutant ES cells have caused the death of all chimeric embryos if the mutation is dominant (or is X or Y linked) and has a very detrimental effect (e.g., *Gata1*; Pevny et al. 1991). Looking at the uteri of the foster mothers shortly after the expected birth date will indicate whether any postimplantation embryonic development took place, because the implantation sites or resorption sites (places at which embryos implanted but then died and were partially resorbed by the uterus) should still be visible as swellings or blood spots on the uterus.

If you suspect that the chimeras are dying, either before or after implantation, try including in the embryo transfer a few wild-type embryos of a distinct coat color along with the chimeric embryos to determine if death is specific to the chimeras. If all of the embryos fail, it is more likely to be the result of a more general problem as detailed in the previous sections. If nonchimeric embryos develop but the ES cell chimeras do not, you might just have a dominant effect. Go to Overview: **Phenotypic Analysis of Dominant Mutant Effects in Mice** (Papaioannou and Behringer 2023c).

Pups Are Born but None Are Chimeric or the Level of Chimerism Is Low

Technique

Were the ES cells kept under optimal conditions during the injection experiment? Trypsinized cells should be held on ice in buffered medium in a capped test tube. Some of these trypsinized cells are transferred into the injection or aggregation dish for chimera production. pH and osmolarity must be correctly maintained in the injection or aggregation dish. Watch the color of the medium and use a sufficient volume of medium in the dish to reduce concerns of evaporation. You can check if this manipulation is compatible with ES cell viability by culturing the "leftover" ES cells after a blastocyst injection or morula aggregation experiment under standard ES cell culture conditions to see if they still grow well.

Are you injecting 12 to 15 ES cells, which seems to be the optimal number for most ES cell lines? Are you confident that your blastocyst injection or morula aggregation procedure is effective and that the ES cells are incorporated into the embryos before transfer to the foster mother? If you think that the lack of incorporation of ES cells might be a problem, you could try injecting or aggregating some marked ES cells (e.g., cells with a *lacZ* or a green fluorescent protein reporter gene); culturing the chimeric embryos overnight; and then looking for the cell marker as an indication of ES cell contri-

bution. If marked cells are not present, it is a good idea to reevaluate your injection or aggregation technique as well as your ES cell–handling technique, because the cells might have died or they might never have been incorporated. If the cells are present in good numbers and appear to be incorporated into the embryo, continue working through the other possibilities.

The Targeted ES Cell Clone Has Lost Developmental Potential

It is a good idea to always check that your parental ES cell line is competent to make chimeras and to contribute to the germline before using it for gene targeting. You can do this yourself by making chimeras with the unmanipulated parental ES cell line or you can depend on information supplied by the person or company from whom you received the ES cells in the first place. If you are sure about the competence of your parental ES cells, try another targeted clone. You should have more than one if you followed our recommendations above. If not, go back and isolate more targeted ES cell clones.

One common reason for a restriction in germline potential is that the ES cells have become aneuploid. If this has happened, a subpopulation of euploid cells might still be in the culture and if the clone is a valuable one (i.e., you have only one targeted clone), it might be worth trying to isolate the euploid cells by subcloning and karyotyping cells (see Chapter 8 in Behringer et al. 2014) and then making chimeras with euploid subclones.

Incompatible Strain Combination

It may be that the host embryo strain is not conducive to making chimeras with your ES cell strain. Search the literature to discover what strains have been successfully used with your particular ES cells. If you are using a compatible combination, try again with larger numbers. If not, consider changing to a different strain of mouse for the host embryos.

Possible Coat Color Phenotype

If offspring are all nonchimeric by coat color, but the sex ratio is skewed heavily in favor of males, consider that the mutation you made may have affected the coat color. Some of the offspring may actually be chimeras, hence the skewed sex ratio, but the coat color is not as expected because of the heterozygous mutation—that is, they are cryptic chimeras. This is fairly unlikely, but it has been known to happen (e.g., *Pdgfra*; see Soriano 1997). The phenotypic sex of XX ⟷ XY chimeras will be skewed in favor of males, assuming that you are using an XY ES cell, because of the nature of sex determination. Genotype some of the offspring to be sure that they are not cryptic chimeras. If they are, you will have an interesting dominant phenotype. Go to Overview: **Phenotypic Analysis of Dominant Mutant Effects in Mice** (Papaioannou and Behringer 2023c). Otherwise, make more chimeras with a different clone.

Could This Be a Phenotype?

Consider the possibility that cells heterozygous for the mutation are incapable of contributing to chimeras because of haploinsufficiency for the gene product, if you made a null mutation, or because of a defective gene product, if your mutation results in a neomorphic or antimorphic protein. If the targeted ES cells grew well, the defect might only become evident as the ES cells differentiate (or try to) in the embryo in vivo. Go to Overview: **Phenotypic Analysis of Dominant Mutant Effects in Mice** (Papaioannou and Behringer 2023c). Keep in mind that if the gene is X or Y linked, the mutation will affect the only copy of the gene in an XY ES cell line.

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