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COMMENTARY

# *Ahr* Null Alleles: Distinctive or Different?

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**ABSTRACT.** Two independent laboratories have generated *Ahr* "null" or "knockout" mice that share some common characteristics but also have distinct phenotypes. In this Commentary, we will discuss our view of the candidate variables that might account for these differences. More importantly, we hope that this discussion can identify important parameters to be assessed by investigators in the process of characterizing their own modified loci. The variables that we have considered include the possibility that different targeting strategies can result in altered products with unsuspected function or that the targeting event itself can alter the function of neighboring genes. Further, genetic background can have an important influence on phenotype, and differences in genome can be introduced during derivation by the type of embryonic stem cells used and by the random segregation of parental genes in the F2 generation of line propagation. In addition, phenotype may be acutely sensitive to environmental variables, such as pathogen and chemical exposure and stress introduced by crowding and disease. Finally, we discuss approaches to resolving differences between null mice and propose a partial solution, the institution of a repository for detailed information on targeted alleles that may not typically be allowed in today's "fast paced" scientific publications. BIOCHEM PHARMACOL **56**;7:781–787, 1998. © 1998 Elsevier Science Inc.

**KEY WORDS.** dioxin receptor; knockout mice; gene targeting; genetic background; cytochrome P450; TCDD

". . .
So oft in theologic wars,
The disputants, I ween,
Rail on in utter ignorance
Of what each other mean,
And prate about an Elephant
Not one of them has seen!"

Moral from the poem *Six Blind Men and the Elephant* by John Godfrey Saxe

## THE PHENOTYPES OF *Ahr* NULL MICE

For nearly 20 years, the AHR† has been known to play a central role in the adaptive metabolic response to PAHs and in the toxicity of halogenated-biphenyls, -dibenzofurans, and -dioxins. Early studies showed that these ubiquitous environmental contaminants can be ligands of the AHR [1, 2]. While it is generally understood that the AHR mediates responses to these chemicals, basic questions remain about the endogenous function of the AHR, such as, whether there is an endogenous ligand, if there are receptor-independent pathways of dioxin toxicity, or whether this protein plays a significant role in mammalian development.

Recent advances in recombinant technology have provided the experimental approach necessary to answer many of the most important questions about AHR biology. Most pertinent to this commentary is the emerging ability to manipulate the murine genome, using homologous recombination or "gene targeting." Over the past few years, this technology has yielded two independently generated *Ahr* "null" or "knockout" mouse strains [3, 4]. In the laboratory of Frank Gonzalez, this inactivation was accomplished using a gene targeting strategy that involved replacement of exon 1 of the *Ahr* locus with a neomycin resistance gene (*Neo*). In addition to insertion of *Neo*, the translational start site for AHR expression was deleted, as well as a stretch of basic amino acids that may play a role in DNA binding (see Table 1). In an independent attempt, our laboratory generated an *Ahr* null mouse, by replacing exon 2 of the *Ahr* gene with a neomycin resistance gene. Exon 2 encodes the basic/helix-loop-helix domain known to be required for Ah receptor nuclear translocator (ARNT) dimerization and DNA recognition. For ease in comparing the two null mice, we have designated the mice generated by the Gonzalez laboratory Δ1/Δ1, because exon 1 of the *Ahr* locus was targeted, and our mice Δ2/Δ2, because we targeted exon 2.

Mice harboring both *Ahr* null alleles exhibit decreased liver size, subtle hepatic portal fibrosis, decreased constitutive expression of certain XMEs, such as cytochrome

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† *Abbreviations: Ahr*, structural gene for the AHR; AHR, aryl hydrocarbon (Ah) receptor; Δ1/Δ1, *Ahr* null lacking exon 1; Δ2/Δ2, *Ahr* null lacking exon 2; ES, embryonic stem; Neo, neomycin resistance gene; PAH, polycyclic aromatic hydrocarbon; RT-PCR, reverse transcriptase-polymerase chain reaction; SPF, specific pathogen free; and XME, xenobiotic metabolizing enzyme.

**TABLE 1. Comparison of the phenotype of two *Ahr* null mice**

| | Δ1/Δ1 | Δ2/Δ2 |
|---|---|---|
| Construct | | |
| ES cells | J1 (129/SvJae) | R1 (129/SvJ × 129/Sv-+$^{p}$+$^{Tyr\text{-}c}$Mgf$^{Sl\text{-}J}$/+) |
| Exon deleted | Exon 1 | Exon 2 |
| Recipient | C57BL/6N | C57BL/6J |
| Lethality | | |
| Intra-uterine | None* | None |
| Neonatal | 40–50% | None |
| Adult | Normal | Normal |
| Growth (1–4 weeks) | Slower | Slower |
| Fertility | Decreased | Decreased |
| Liver | | |
| Size | 50% smaller (4 weeks)† | 25% smaller (3 weeks) (persists through life) |
| Lobule structure | Normal | n/a |
| Gross pathology/Histology | Portal tract fibrosis (3 weeks) | Pale, mottled, spongy (1 week/2 weeks) |
| | Centrilobular hypercellularity (3 weeks) | Extensive microvesicular fatty metamorphosis of hepatocytes (1 week/2 weeks) |
| | Inflammation of bile ducts (3 weeks) | Prolonged extramedullary hematopoiesis (1 week/3 weeks) |
| | Eosinophilia (3 weeks) | Mild portal region fibrosis (2 weeks) |
| | Glycogen depletion (3 weeks) | |
| P450 expression/activity | | |
| Inducible | | |
| Cyp1a1 | Not inducible | n/a |
| Cyp1a2 | Not inducible | Not inducible |
| Constitutive | | |
| Cyp1a2 | 10% expression | 25% expression |
| Ugtl*06 | 15% expression | n/a |
| Inducible EROD activity | n/a | Not inducible |
| AHR expression | | |
| Protein | n/a | Not present |
| mRNA | Not present | Present at predicted size |
| Immunology | | |
| Lymphoid histology | Smaller PALS‡ (4 weeks) | Normal |
| Splenocyte numbers | 20% (2–3 weeks) | Normal (2–3 weeks) |
| | | 150% (6 weeks)§ |
| | Normal (10–12 weeks) | |
| | 50% (25–32 weeks) | |
| Lymphocyte subset proportions | | |
| Spleen | Normal | Normal |
| Thymus | Normal | Normal |

*Characteristics of *Ahr* null mice are given in comparison with *Ahr*$^{+/+}$ littermate controls.

†Age of mice at the time of the observed pathology is indicated in parentheses. When pathology is observed at several time points, the range is indicated by a dash (-). If pathology is observed to resolve with time, disappearance is indicated as denominator (/x).

‡Periarterial lymphatic sheaths.

§Reported for some but not all mice.

P4501A2, and decreased body size over the first 4 weeks of age relative to their littermate controls (Table 1). The *Ahr* null mice from both laboratories are also resistant to the XME induction that is classically observed in response to dioxin exposure (e.g. P4501A1). These observations are in keeping with the known role of AHR in induction of certain XMEs and also suggest an additional role in controlling their constitutive expression. Perhaps of greater interest is the consistent observation that AHR is important to liver development, possibly affecting both its function and size throughout life.

Unexpectedly, there are also differences in the reported phenotypes of the null mice generated by the two laboratories. For example, significant mortality is observed among Δ1/Δ1 pups within the first 2 weeks of age, whereas survival of Δ2/Δ2 neonates is similar to wild-type littermate con-

trols. Furthermore, livers of Δ1/Δ1 animals exhibit several pathologies that are not observed in Δ2/Δ2 animals, including eosinophilia of periportal hepatocytes, glycogen depletion, and inflammation of bile ducts. In addition, the degree of hepatic portal fibrosis of Δ1/Δ1 animals may also be more severe than that of Δ2/Δ2 mice. Further, there are differences between the spleens of the *Ahr* null mice. Perinatal Δ1/Δ1 *Ahr* null mice contain about one-fifth fewer splenocytes than littermate controls. Splenocyte numbers increase in these mice to levels comparable to those in control mice by about 8 weeks. Yet, there is a subsequent downward trend, such that by 25–32 weeks of age, there is again a 50% reduction. Further, there are fewer lymphoid cells in the peripheral lymph nodes of these mice. In contrast, Δ2/Δ2 spleens contain, albeit sporadically, more splenocytes at 6 weeks of age than their littermate controls. Another reported difference between the two models is that the livers of Δ2/Δ2 neonatal mice exhibit prolonged extramedullary hematopoiesis and extensive microvesicular fatty metamorphosis within hepatocytes, particularly acute 1 week after birth and completely resolved by 4–5 weeks of age. This particular liver phenotype was not reported for Δ1/Δ1 mice.

## ADDRESSING DISCREPANCIES BETWEEN MOUSE MODELS

At first look, the descriptions of the *Ahr* Δ1/Δ1 and Δ2/Δ2 null animals suggest that these two mouse lines are fundamentally different. More to the point, one can take the cynical view and suggest that one (or both) of these alleles is not a complete null or that one (or both) of the targeting events affects expression of a nearby locus. Another interpretation of these same observations is more optimistic. That is, there are unknown environmental or genetic factors that influence the penetrance or presentation of the *Ahr* null phenotype, and that the identification of these variables will help us to gain insights into AHR biology. From cynical to Pollyannaish, one thing is clear: we need to ascertain why these phenotypes are different to understand AHR biology. Therefore, we would like to address factors that might affect the phenotypes of *Ahr* null mice, including gene targeting strategies, genetic backgrounds, and environmental variables.

## TIMING

It is possible that the reported phenotypic differences between null mice reflect the timing of the observations made in each laboratory rather than disparities in genotype or environment. If studies of null mice are conducted at different ages, then transient phenotypes may be missed and misinterpreted as absolute. Most certainly, different laboratories do not always study their models at the exact same time points. In our case, livers of Δ2/Δ2 mice exhibit a pale and mottled gross pathology at 1 week of age, which resolves within a week or two. Although this phenotype is not observed in Δ1/Δ1 mice, it may be that liver histology was not assessed at 1 week of age. Other observations, such as changes in relative spleen size or cellularity, are less likely to result from discrepancies in the time of observations during development, because both laboratories have assessed spleens at several similar time points.

## TARGETING STRATEGIES

As described above, different strategies were implemented to render null alleles at the *Ahr* locus. In the Δ1/Δ1 mouse, the translational start site of *Ahr* is removed, whereas the null allele of our Δ2/Δ2 mouse contains both the transcriptional and translational start sites. Despite the careful planning that went into the development of these mouse models, it is possible that the targeted gene of either mouse expresses a truncated product with unpredicted biological consequences. To address this possibility, we used western blot analysis with domain specific antisera to detect AHR expression in liver cytosol. Further, we used the photoaffinity ligand, 2-azido-3-[$^{125}$I]-iodo-7,8-dibromodibenzo-*p*-dioxin, and were unable to detect a functional receptor in null cytosols [5]. There are, however, limitations to this approach. Most important is that a functionally relevant amount of protein might be expressed at a level that is below the detection limit (about 5% of ambient liver AHR levels). Thus, a decrease in AHR expression might be mistaken for complete loss of expression or function. Further, Western blotting works optimally when the size of the protein to be identified is known. If only a portion of the targeted *Ahr* allele is transcribed, investigator bias may misinterpret important truncated species as nonspecific.

Another means of determining if there is a functional product is to measure expression of the relevant mRNA species by RT-PCR. This method has the advantage of being highly sensitive to low level expression. In the case of our Δ2/Δ2 mouse, cloning and sequencing of the RT-PCR amplification products indicate that mRNA is expressed, but at a decreased level, and that exon 1 is spliced to exon 3. This splicing event was predicted in our design of the targeting construct and results in a frame shift and termination codon 7 residues past the splice junction. This splice variant could express a peptide of 23 amino acids and, although unlikely, this peptide could have biological activity. Similar analysis for the presence of transcript in Δ1/Δ1 *Ahr* null mice by RT-PCR was used to suggest that there is no expression of a partial null or mutant protein [6]. We take issue with this conclusion, since primers were designed to anneal to sequences of exons 1 and 2. As exon 1 is replaced with *Neo*, this PCR amplification strategy would inherently fail to identify any novel transcript from that null allele.

While differing knockout strategies may target the same gene, they might also affect neighboring genes. Insertion of selectable marker genes in the targeted allele might alter the function of neighboring genes, since their sequence might influence local DNA structure and neighboring gene expression. For example, differences in PGK-*Neo* insertion

to generate *Mrf4* knockout mice differentially affect the function of neighboring genes [7]. Thus, it is possible that one (or both) of the targeting strategies used to create *Ahr* mice alters the function of neighboring genes. Adjacent DNA might encode for a molecule that compensates for lost signal transduction by the AHR or encode for a molecule critical in lymphocyte homing or hepatic fat metabolism during development. Because the region of chromosome 12 near the *Ahr* locus is poorly characterized [8], we do not yet know if neighboring genes could be responsible for the phenotype reported for either null allele.

## GENETIC BACKGROUNDS AND THE VALUE OF CONGENIC MICE

Differences between the phenotypes of the *Ahr* null mice could also be related to the influences of distinct genetic backgrounds, which can vary significantly even when identical gene targeting strategies are employed. To generate both the Δ1/Δ1 and Δ2/Δ2 animals, the targeting construct was introduced into mammalian ES cells, which then were incorporated into blastocysts. Next, these chimeric blastocysts were implanted in pseudo-pregnant females and grown to adulthood. Of the chimeric offspring, those that carried the targeted gene in their germinal cells were bred to generate knockout animals. ES cells for both sets of knockout mice are derived from the 129 mouse, which might indicate that we can consider these ES cells to be genetically identical. However, there are several substrains of 129 that are unusually divergent [9], such that ES cells generated from these various substrains can have distinct genetic backgrounds. The Δ1/Δ1 *Ahr* null mice are generated from J1 ES cells, which are derived from the "steel" group of the 129 substrains (129/SvJae). The R1 ES cell line that is used to generate the Δ2/Δ2 *Ahr* null mouse is derived from blastocysts carrying genes of both the "parental" and "steel" substrains (129/SvJ × 129/Sv-$+^{p}$ + $^{Tyr\text{-}c}$Mgf$^{Sl\text{-}J}$/+). Most 129 substrains, including those used to derive R1 and J1 ES cells, are polymorphic at many loci and lack, for example, histocompatibility due to differences in minor histocompatibility loci. There is an excellent review of the origins of ES cells and 129 substrains by Simpson *et al.* [9]. A more subtle point is that, in theory, genetic variability may also result from mutations that occur during ES cell derivation and repeated culturing. Thus, even if transgenic mice were derived from a common ES cell stock, they might not have identical genetic backgrounds.

The last step in the generation of the knockout mice introduces, perhaps, the greatest degree of genetic variability. For both Δ1/Δ1 and Δ2/Δ2 *Ahr* null mice, founder mice are chimeras that carry 129-derived germ cells. For these mice, germ cells carry the entire genetic background of the ES cell along with the targeted allele. Assuming, but only for the sake of simplicity, that the ES-derived germ cells of the chimera are homozygous at all other loci, then meiosis will result in identical gametes, regardless of the different patterns of gene segregation. In addition, the progeny of the intercross between the chimera and the recipient inbred strain (commonly C57BL/6) results in identical F1 offspring, carrying one chromosome from each parent (Fig. 1). To obtain mice that are homozygous for the null allele, these F1 offspring are brother–sister mated, resulting in F2 progeny that carry an equal proportion of alleles from the chimera and its mating partner. However, meiotic events result in a random segregation of parental genes that are no longer identical on both chromosomes of the F2 offspring. The result is that very different combinations of genetic backgrounds can exist between individuals, even within a given litter (Fig. 1). Thus, if each parent provides different alleles that either compensate for the lost function of the *Ahr* locus or that alter the phenotype of the null mouse by a different mechanism, then different combinations of these alleles will be present in each of the F2 mice (Fig. 1). Some mice within this litter might carry entirely different genetic backgrounds (mouse A vs G), and exhibit very different phenotypes. The divergent genetic backgrounds of

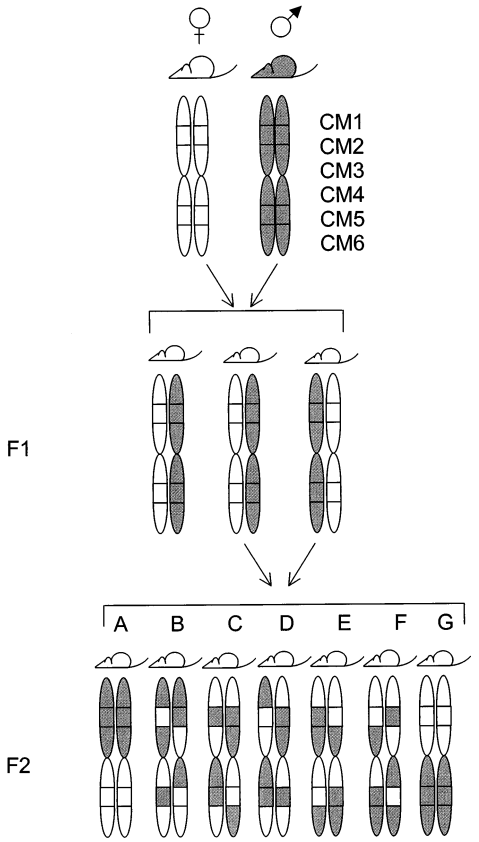


**FIG. 1. Hypothetical differences in the genetic backgrounds of F2 mice. When a female mouse of one strain (white) is mated with a male of a different strain (gray), each F1 littermate carries an identical combination of parental backgrounds. However, when F1 mice are inbred, the genetic backgrounds of the F2 generation can be highly divergent. Six hypothetical polymorphic genes (CM1 through CM6) are shown below each littermate which could compensate for the loss of AHR expression or otherwise modify its phenotype. For each mouse, a subset of possible segregations of alleles from both strains is presented.**

these mice virtually assures that the inbred lines generated from repeated incrossing of these founders will result in strains that carry unique segregations of parental genes that have the potential to influence the phenotype of the knockout animal.

Effects of genetic background on the phenotype of knockout mice are well documented, including mice carrying targeted transforming growth factor β1 [10] and epidermal growth factor receptor [11, 12] loci. Genes that affect dioxin toxicity, such as the hairless locus (*hr*), have been identified [13, 14], and there is potential for polymorphic loci to affect the phenotype of the *Ahr* null mice. Certainly, differences in genes that encode detoxification enzymes, hormones, and aspects of immune responses may be important in altering responses to modified AHR [15]. To overcome potential genetic background effects that are introduced by differences in ES cells and by the random segregation of parental genes, congenic mouse strains can be produced by repeated backcrossings to a recipient inbred strain, selecting for the locus of interest at each generation. Congenic mice differ from the recipient parent only at the region of the selected locus. Progeny from each breeding that carry the targeted allele are identified and are then backcrossed again with the designated strain. For example, currently in our laboratory, the targeted *Ahr* null allele is being selected during successive backcrosses to the C57BL/6J mouse. Each backcross reduces residual heterozygosity by 50%. By 20 backcrosses, the congenic mouse contains the targeted *Ahr* null allele on a background that consists almost entirely of the C57BL/6J alleles. Even by 10 backcrosses, strains that carry the *Ahr* null alleles are 99.8% identical with C57BL/6J at all other loci.

The above approach must be distinguished from a different strategy that reduces heterozygosity through successive brother–sister matings. Inbreeding generates homozygosity at a slower rate than backcrossing to a congenic strain. For example, it would take 30 generations of inbreeding to generate mice in which the genome is 99.8% homozygous, relative to 10 generations of backcrosses for congenics [16]. In addition, the genetic background of inbred mice will still be largely unknown, and the only appropriate controls will be littermates. Both of these strategies reduce the background genetic variability, and allow for the generation of appropriate "wildtype" controls. Congenic strains developed for both *Ahr* null alleles would help to take advantage of disparities in phenotypes of null mice, serving to avert the potential for controversy and generate useful data for identification of important compensating or modifying genes. Unfortunately, true congenics can take up to 3 years to develop and require that the targeted mice (e.g. *Ahr* Δ1/Δ1 and Δ2/Δ2) are bred onto the same backgrounds.

## XENOBIOTICS AND INFECTIOUS AGENTS IN THE ENVIRONMENT

Another source of variability is the housing environment of the null mice. Several environmental factors might affect the phenotype of an *Ahr* null animal. For example, differences between the liver phenotypes of the Δ1/Δ1 and Δ2/Δ2 *Ahr* null mice might result from different degrees of exposure to ubiquitous toxic compounds. Without a functional AHR, null mice might not be able to biotransform and excrete these compounds, and may be more susceptible to low level contamination. For example, early studies have suggested the presence of AHR agonists in animal diets [17] and in bedding materials [18]. If the AHR and the related increased activity of XMEs protect mice from the toxicity of PAH-like components, then *Ahr* null mice might be more sensitive to these variables and display disparate phenotypes under different housing conditions. We tried to address this possibility by comparing the phenotype of our Δ2/Δ2 *Ahr* null mice maintained on wood chip bedding and standard laboratory chow with that of mice maintained on corncob bedding and a purified synthetic diet. Regardless of food and bedding regimens, smaller liver size and hepatic fatty metamorphosis were seen to the same extent among the *Ahr* null mice [4]. Finally, more mundane parameters of housing may have a significant influence on phenotype. For example, the number of animals per cage may be important, especially given that *Ahr* null mice can be smaller than littermate controls and may be more susceptible to sibling aggression or parental culling. This could result in loss of neonates in the first 2 weeks of life. Moreover, the level of stress could result in novel phenotypes.

There are several disparities in immune system parameters between the two null mice. The Δ1/Δ1 *Ahr* null mice exhibit eosinophilia, which is often indicative of parasitic infection, yet they have relatively fewer splenocytes. Conversely, the Δ2/Δ2 *Ahr* null mice do not have eosinophilia, but exhibit sporadic splenic hypercellularity, which might also indicate infection. It is possible that the AHR is involved in the regulation of immune responses to pathogens. This hypothesis is supported by studies that show that immune responses are highly sensitive to dioxin exposure [19–22]. If infection can differentially alter the phenotype of *Ahr* null animals, then there is an imperative to reduce the exposure of these mice to pathogens. This problem is exacerbated by the fact that there are many pathogenic viruses (e.g. Minute Virus of Mice, Mouse Hepatitis Virus, Sendai Virus, and Mouse Encephalomyelitis Virus), bacteria (e.g. Mycoplasma spp.), and parasites (e.g. pinworms and mites) that are known to infect captive mice.

Precautions can be taken to reduce pathogen exposure. Animals can be handled in a manner that minimizes the introduction of new infectious agents, involving use of sterile microisolator cages, food and water, and sterile manipulation of animals in a laminar flow hood. Further, animals can be routinely checked for pathogens and be used for experiments only if they are SPF. Infectious agents that are screened by our laboratory include Mouse Hepatitis Virus, Sendai Virus, Pneumonia Virus of Mice, Parvovirus, Theiler's Murine Encephalomyelitis Virus, Reovirus Type 3, Lymphocytic Choriomeningitis Virus, Ectromelia, Rotavirus, Mouse Adenoviruses 1 and 2, Polyoma Virus, *Enceph-*

**TABLE 2. "Strawman" criteria for discussion**

| |
|---|
| Gene targeting strategy |
| ES cells |
| Exon deleted |
| Recipient |
| Expression of targeted allele |
| Protein, RNA |
| Status of congenic mouse |
| Background mouse strain |
| Number of backcrosses |
| Status of pathogens and pollutants in environment |
| Bedding |
| Food |
| Water |
| Whole organism evaluation |
| Lethality: intra-uterine, neonatal, adult |
| Growth |
| Fertility |
| Gross pathology |
| Evaluation of cells that express high levels of the gene of interest |
| Histology |
| Function/Physiology |

*alitozoon cunculi*, *Mycoplasma pulmonis*, and Cilia Associated Respiratory Bacillus. However, a SPF mouse is not synonymous with a sterile or "germ free" mouse, lacking all infectious agents. There are a large number of infectious agents that commonly infect mice and have no pathogenicity in immunologically healthy animals. In fact, SPF mice can carry a variety of microorganisms and unknown pathogens. Therefore, null animals ought to be maintained as aseptically as possible, and studies should not assume that animals are free of infectious agents. If there are reasons to suspect that the immune system of the null animal is compromised, then perhaps, side-by-side comparisons of *Ahr* null mice that are housed in the same facility might be required to determine if exposure to different infectious agents results in disparate mouse phenotypes.

## CONCLUSIONS

The reported differences in *Ahr* Δ1/Δ1 and Δ2/Δ2 alleles may emerge as an example of the rule rather than the exception among gene-targeted animals. Thus, results from future gene-targeting experiments may be more readily interpretable if all investigators that are using this technology reach a consensus with regard to the parameters that should be reported. Unfortunately, the trend toward rapid publication, combined with editorial policies that invoke strict page limitations, may hamper our progress in this important area. Ideas open for discussion should include proposals for a common background strain, a method to report environmental variables (e.g. definitions of "specific pathogen free" and dietary composition). We have provided a "strawman" set of criteria (Table 2) to initiate discussion. This list would provide information about the gene-targeting strategy and expression of the targeted allele, including determination of transcriptional and translational products of the targeted locus. Perhaps we can create a website on the Internet as a repository for this more mundane, but necessary, information. By whatever means we use, it is essential for us to agree upon common aspects in null mouse assessments. Thereby, we are more likely to study the same elephant or, rather, the same mouse.

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