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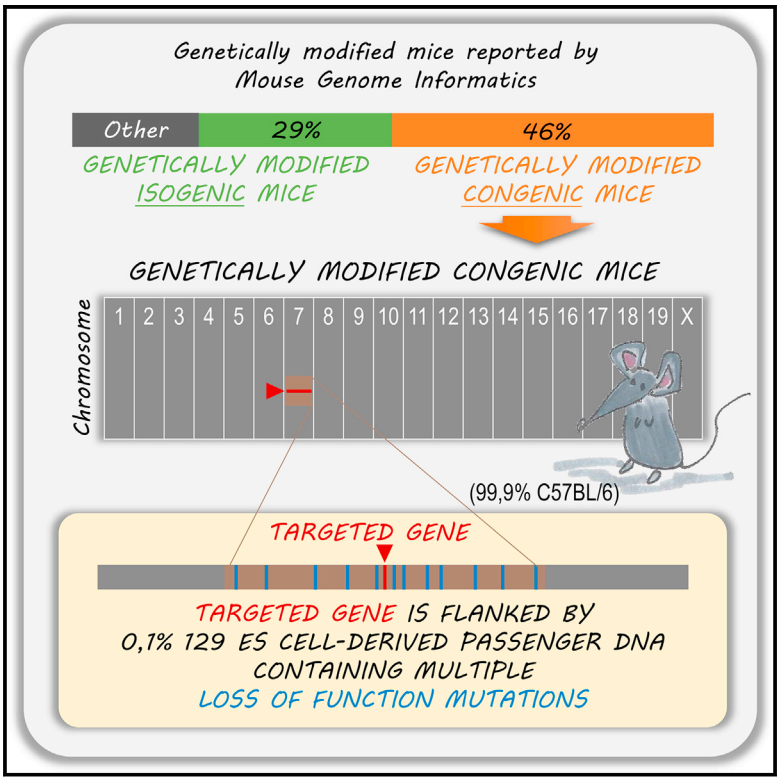
Passenger Mutations Confound Interpretation of All Genetically Modified Congenic Mice

Graphical Abstract



Highlights

- All genetically modified congenic mice are populated with passenger mutations
- Phenotypic outcome of genetically modified congenic mice is potentially incorrect
- Phenotypic interference of passenger mutations is illustrated by some case studies
- A webtool provides a list of passenger mutations affecting genetically modified mice

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In Brief

The importance of genetic background in genetically modified congenic mice is well known. Vanden Berghe and Vandenabeele and colleagues predict through comparative genomics (webtool) that nearly all 129-derived genetically modified congenic mice are affected by multiple inactivating passenger mutations despite intensive backcrossing and illustrate phenotypic interference through some case studies.

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Passenger Mutations Confound Interpretation of All Genetically Modified Congenic Mice

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SUMMARY

Targeted mutagenesis in mice is a powerful tool for functional analysis of genes. However, genetic variation between embryonic stem cells (ESCs) used for targeting (previously almost exclusively 129-derived) and recipient strains (often C57BL/6J) typically results in congenic mice in which the targeted gene is flanked by ESC-derived passenger DNA potentially containing mutations. Comparative genomic analysis of 129 and C57BL/6J mouse strains revealed indels and single nucleotide polymorphisms resulting in alternative or aberrant amino acid sequences in 1,084 genes in the 129-strain genome. Annotating these passenger mutations to the reported genetically modified congenic mice that were generated using 129-strain ESCs revealed that nearly all these mice possess multiple passenger mutations potentially influencing the phenotypic outcome. We illustrated this phenotypic interference of 129-derived passenger mutations with several case studies and developed a Me-PaMuFind-It web tool to estimate the number and possible effect of passenger mutations in transgenic mice of interest.

INTRODUCTION

The importance of the genetic background in the phenotype of transgenic mice is well established (Gerlai, 1996; Simpson et al., 1997). Until recently, genetically modified mice were mostly created using germline transmission competent embryonic stem cells (ESC) lines derived from the 129 strain of mouse (Simpson et al., 1997). For phenotypic studies, chimeric mice are backcrossed repeatedly to a particular strain, often C57BL/6J (Lusis et al., 2007). Although this strategy is commonly considered to omit ESC-derived genetic background effects, the region closely flanking the targeted gene remains of donor origin (passenger genome) because the gene recombination frequency decreases near the targeted locus. The genetic variation between donor and recipient strains implies that the passenger genome typically contains mutations in the regions flanking the targeted gene (passenger mutations). This might influence phenotypic outcomes in genetically modified mice and cells derived thereof (Lusis et al., 2007).

When mice are backcrossed for 10 generations to C57BL/6J (certified as congenic mice; Flaherty, 1981) the probability that a 1 centiMorgan (cM) region flanking each side of the targeted gene is still of donor origin is 0.91 (Lusis et al., 2007). In congenic mice, the linkage between a targeted gene and flanking donor genes increasingly fades away from 10 cM onward (Lusis et al., 2007). This problem has been clearly highlighted back in the mid-nineties (Crawley et al., 1997; Gerlai, 1996; Simpson et al., 1997). Although several reports identify 129-derived protein coding variants in for example DAP12 (*Tyrobp*) signaling (McVicar et al., 2002), DNA polymerase iota (*Poli*) (McDonald et al., 2003), beaded filament structural protein 2 (*Bfsp2*) (Alizadeh et al., 2004; Sandilands et al., 2004), disrupted-in-schizophrenia (*Disc1*) (Gómez-Sintes et al., 2014; Koike et al., 2006), apolipoprotein A-II (*Apoa2*) (Su et al., 2009), oculocutaneous albinism II (*Oca2*) (Rogers et al., 2012), and chromatin licensing and DNA replication factor 1 (*Cdt1*) (Coulombe et al., 2013), this issue has been generally ignored. This issue however re-emerged when *Casp1* null mice were found to carry an inactivating passenger mutation in the neighboring *Casp11* gene (Kayagaki et al., 2011). Essentially, the strong protection observed in *Casp1* null mice against a lethal lipopolysaccharide (LPS) challenge was found to be mainly due to this inactivating passenger mutation in the *Casp11* gene (Kayagaki et al., 2011). This raised again the awareness of a potential phenotypic effect of closely linked passenger mutations originating from the 129-derived ESCs. This is unfortunately not an isolated case, because other genetically modified mice are also affected by this *Casp11* passenger mutation such as *Casp3*- and *Birc2* null mice (Kenneth et al., 2012; Vanden Berghe et al., 2013). This prompted us to setup a comparative genomics analysis

between C57BL/6 and 129 strains and map these differences to all genetically modified mice that origin from 129-strain ESCs reported by the Mouse Genome Informatics (http://www.informatics.jax.org/).

RESULTS

Comparative Genomics of the Reference Genome and 129 Strain Genomes

To analyze the impact of inactivating passenger mutations on 129 ESC-derived genetically modified mouse strains, we compared the 129 and C57BL/6J genomic sequences released by The Wellcome Trust Sanger Institute (Keane et al., 2011; Yalcin et al., 2011). Filtering on predicted protein sequence alterations yielded 949 indels and 446 single nucleotide polymorphisms (SNPs) affecting 1,084 genes (Figure S1A). Note that the number of indels and SNPs that affect protein sequence relative to the C57BL/6J reference sequence in several other laboratory inbred strains are similar to the number observed in the 129 strain, while a higher number is observed in wild-derived inbred strains (Table S1). Of these predicted protein sequence alterations in the 129 strain, 188 result in gained or lost STOP codon (13%); 875 result in frameshift variants, in frame indels or coding sequence variants (63%); and 332 splice donor or acceptor variants (24%).

Annotation of the 129-Derived Protein Coding Variants to Genetically Modified Congenic Mice

The probability and number of passenger mutations in genetically modified congenic mice typically depends on the size of the considered flanking region around the target gene (Figure 1A). The international database resource for the laboratory mouse "Mouse Genome Informatics" (MGI) illustrates that within the pool of genetically modified mice (anno January 2015): 58% are systemically modified, 29% are reporter mice, and 13% are conditionally modified mice (Figure 1B). Almost 8,000 genetically modified mice (corresponds to 80%) of the total pool are derived from 129 ESCs (Figure 1C). By annotating the found passenger mutations to the 129-derived genetically modified mouse lines, we found that 99.5% of these mouse lines are affected by a median number of 20 passenger mutations within a 10 cM flanking region (Figures 1E and S1B). Decreasing the size of the region flanking each side of the target gene to 5 or 1 cM, which increases the probability to respectively 0.63 and 0.91, reveals that still 97% (Figure 1F) and 71% (Figure 1G) of the reported 129 ESC-derived genetically modified congenic mice are affected by a median number of 11 and 2 passenger mutations, respectively (Figures S1C and S1D, Table S2). This implies that nearly all 129-derived genetically modified congenic mice contain multiple passenger mutations despite intensive backcrossing. Consequently, the phenotypes observed in these mice might be due to flanking passenger mutations rather than a defect in the targeted gene. Note that 76% of these reported 129-derived genetically modified congenic mice are affected by a passenger mutation that results in a gained STOP codon (Table S2).

Annotation of the 129-Derived *Casp11* Passenger Mutation to Genetically Modified Congenic Mice

As a reference locus, we analyzed the recently identified 129-derived *Casp11* inactivating passenger mutation. There are 294 genes located within a 10 cM region flanking each side of the *Casp11* gene. To date, 86 genetically modified mouse lines (derived from 129 ESCs) are reported of which the targeted gene resides within this *Casp11* flanking genomic region. This indicates that in addition to the reported *Casp1* (Kayagaki et al., 2011) and *Birc2* null animals (Kenneth et al., 2012), at least 84 other 129-derived genetically modified congenic mice could be carrying the reported inactivating *Casp11* mutation (Table S3), which consequently would make them resistant to LPS-induced shock (Hagar et al., 2013; Kayagaki et al., 2011; Vanden Berghe et al., 2013; Wang et al., 1998). *Casp12* flanks most closely to *Casp11*, and therefore *Casp12* null mice are expected to carry this *Casp11* inactivating passenger mutation, which could explain their resistance to LPS-induced shock (Saleh et al., 2006).

The 129-derived *Casp11* Passenger Mutation Confounds Phenotypic Interpretation of Genetically Modified Congenic Mice Targeting Several Members of the Matrix Metalloproteinase Family

We noticed that several MMP family members reside within <5 cM distance of *Casp11* (Table S3). Considering that *Mmp7*-, *Mmp8*-, and *Mmp13* null mice are protected against LPS lethality (Vandenbroucke et al., 2013; Vandenbroucke et al., 2012; Vandenbroucke et al., 2014), we analyzed the contribution of the *Casp11* inactivating passenger mutation in this phenotype. The closest *Mmp* gene to *Casp11* is *Mmp13* (<1 cM). We found that the *Mmp13* null mice (backcrossed ≥10 generations to C57Bl/6) originating from Stephen M. Krane's lab and subsequently bred in our lab (C.L., $Mmp13^{Cli-/-}$ mice) (Vandenbroucke et al., 2013) contain the *Casp11* mutation despite intensive backcrossing to C57BL/6 (Figures 2A and 2B). Moreover, the $Mmp13^{Cli-/-}$ mice were protected against death (Figure 2C) and hypothermia (Figure 2D) caused by *S. enterica*-derived LPS. The resistance to LPS was similar as observed in *Casp11* null mice (Figures 2G and 2H). However, *Mmp13* null mice from Stephen M. Krane's lab that were bred in E.B. Brown's lab ($Mmp13^{Ebb-/-}$ mice) lost the *Casp11* mutation due to extensive additional backcrossing (Figures 2A and 2B). Essentially, these $Mmp13^{Ebb-/-}$ mice were not protected against a lethal LPS dose, as shown by survival rates (Figure 2D) and body temperature (Figure 2E). Using a sub lethal dose of LPS neither resulted in any protection in these $Mmp13^{Ebb-/-}$ mice (Figure S2A), while showing even an increased hypothermic response (Figure S2B). A more extended analysis in different MMP-deficient mice revealed a significant association ($p = 0.048$, Fisher's exact test) between the presence of the *Casp11* mutation and MMP-deficient mice that reside on chromosome 9 (Figures S2C and 2D). Similarly, analysis of LPS resistance of several MMP-deficient animals revealed a significant association ($p = 0.029$, Fisher's exact test) between LPS-resistance and the presence of the mutated *Casp11* allele on chromosome 9 (Figures S2E–S2I). Taken together, our data indicate a phenotypic interference of the mutated *Casp11* allele with several members of the MMP family. That said, we do not exclude a possible role of MMPs in septic shock, because specific MMP8 inhibitors and broad-spectrum MMP inhibitors improve the outcome in models of

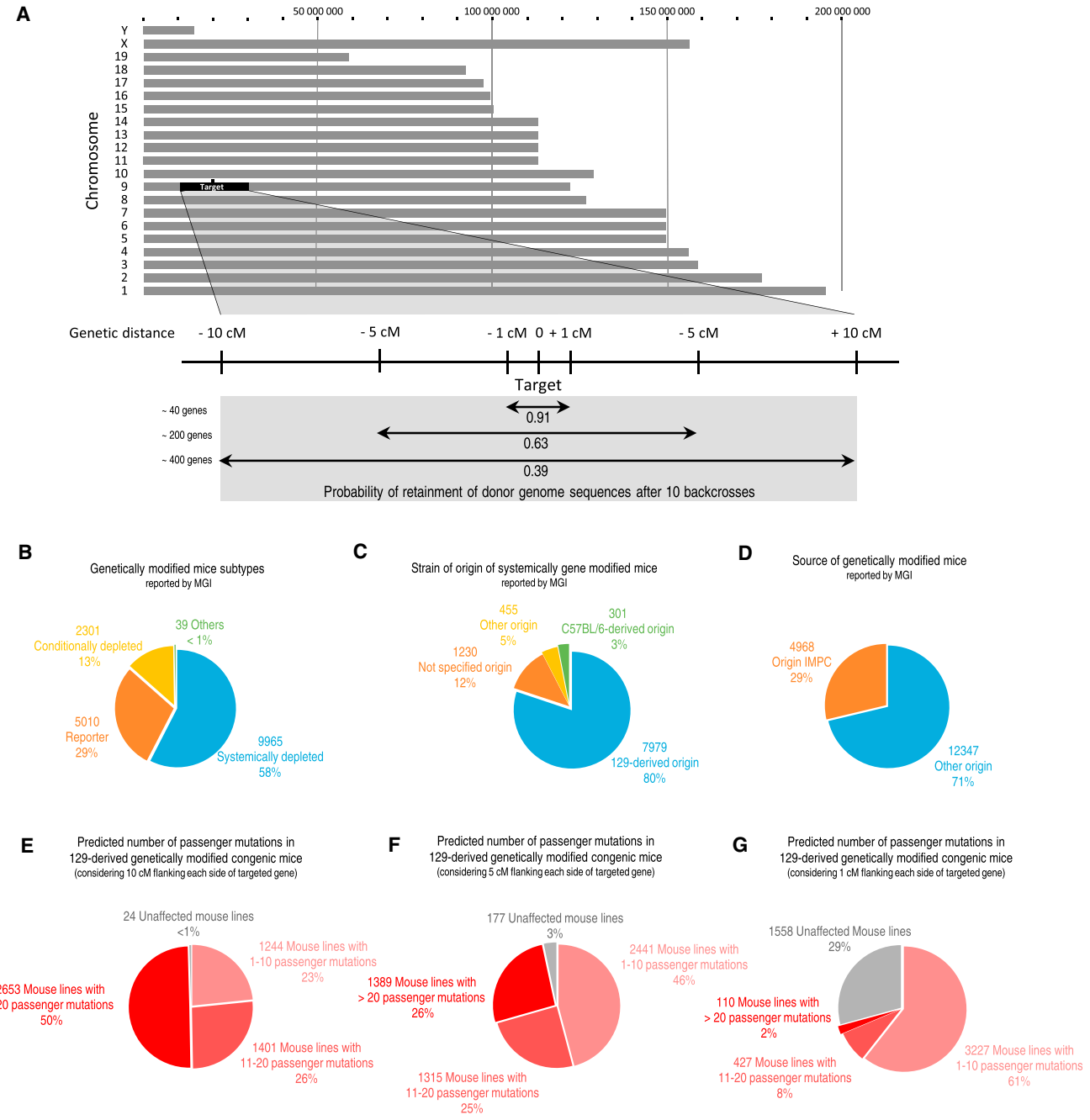

Figure 1. All Genetically Modified Congenic Mice Originating from 129 Strains Are Populated with Passenger Mutations Despite Intensive Backcrossing to C57BL/6

(A) Schematic representation of the probability that the region flanking the gene targeted by transgenic approaches remains of donor origin (source of embryonic stem cells). For a region of 1 cM flanking the targeted gene in mice that were backcrossed 10 times to C57BL/6, the probability that this region remains of donor origin is 0.91 (Flaherty, 1981; Lusis et al., 2007). Flanking regions of 5 and 10 cM have 0.63 and 0.39 probability, respectively, of remaining donor origin.

(B) Analysis of the classification of genetically modified mice reported by Mouse Genome Informatics anno January 2015.

(C) Analysis of the strain of origin of systemically gene modified mice reported by Mouse Genome Informatics anno January 2015.

(D) Analysis of the source of genetically modified mice reported by Mouse Genome Informatics (situation on January 2015).

(E–G) Analysis of the number of 129-derived passenger mutations per genetically modified congenic mouse strain for a genomic region of 10, 5, or 1 cM flanking both sides of the targeted allele based on sequences released by The Wellcome Trust Sanger Institute (Keane et al., 2011; Yalcin et al., 2011). All pie charts indicate absolute and relative amounts. MGI, Mouse Genome Informatics; IMPC, International Mouse Phenotyping Consortium. See also Figure S1 and Tables S1 and S2.

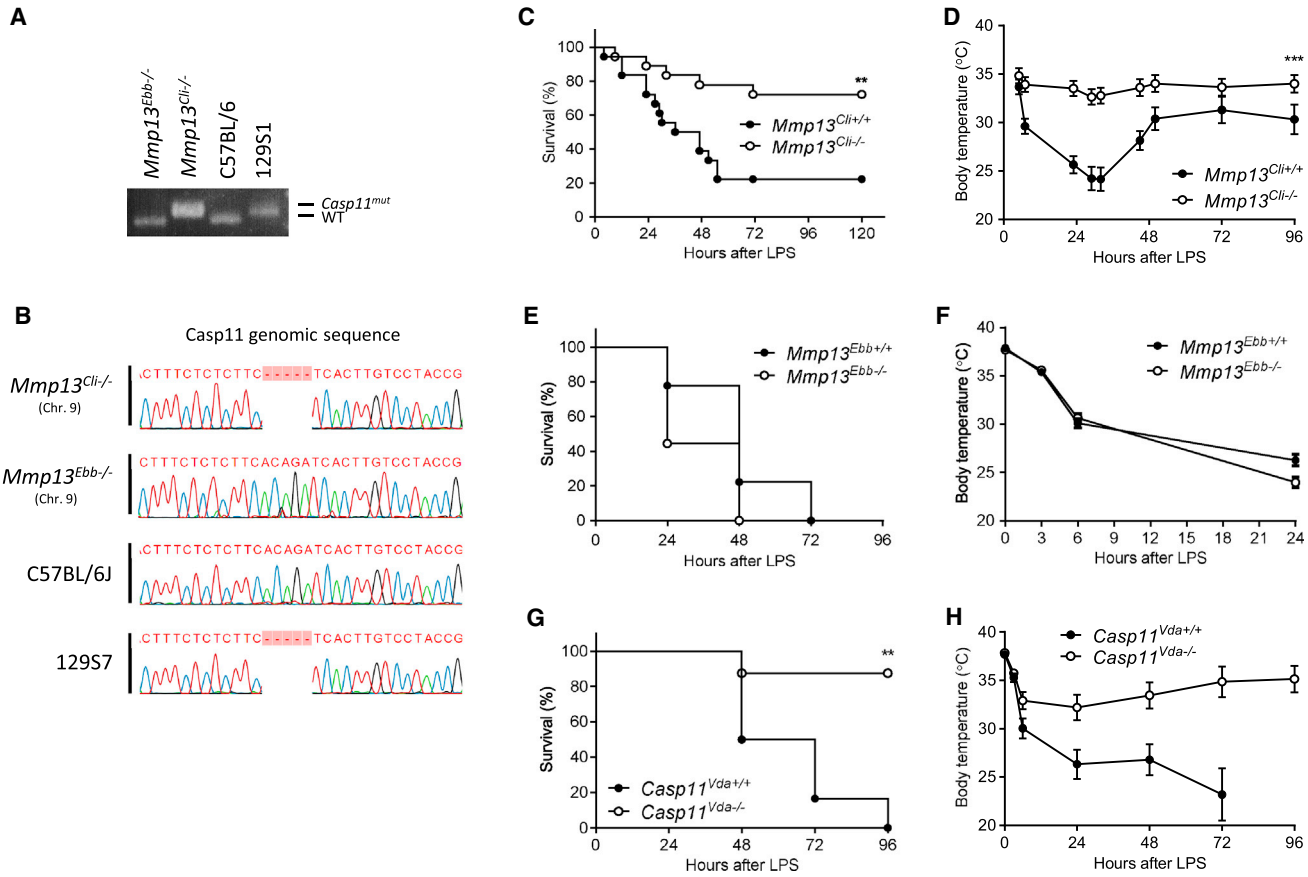

Figure 2. *Casp11* Inactivating Passenger Mutation Provides *Mmp13* Null Mice Resistance to LPS-Induced Shock

(A) Electrophoresis-separated PCR fragments of the genomic DNA region flanking the 5-bp deletion in *Casp11* gene in *Mmp13* null mice derived from C.L.'s lab ($Mmp13^{Cli-/-}$), which carry the *Casp11* passenger mutation, or from E.B. Brown's lab ($Mmp13^{Ebb-/-}$), which have the wild-type *Casp11* gene. Samples derived from C57BL/6 and 129S7 strains were included as negative and positive controls, respectively.

(B) DNA-sequencing profiles of the genomic DNA region flanking the 5-bp deletion in *Casp11* gene in $Mmp13^{Cli}$ and $Mmp13^{Ebb}$ null mice. C57BL/6 and 129 strains were used as negative and positive controls, respectively, for the 5-bp deletion in *Casp11*.

(C and D) Survival and body temperature were analyzed in function of time after intraperitoneal injection of 17.5 mg kg^{-1} *Salmonella enterica* LPS in $Mmp13^{Cli-/-}$ mice (n = 18) and in matching wild-type control $Mmp13^{Cli+/+}$ mice (n = 18). The combined results of two independent experiments are shown.

(E and F) Survival and body temperature were analyzed in function of time after intraperitoneal injection of 7 mg kg^{-1} *S. enterica* LPS in $Mmp13^{Ebb-/-}$ mice (n = 9) and matching wild-type control $Mmp13^{Ebb+/+}$ mice (n = 9).

(G and H) Survival and body temperature were analyzed in function of time after i.p. injection of 7 mg kg^{-1} *S. enterica* LPS in *Casp11* null mice ($Casp11^{Vda-/-}$, n = 8) and matching wild-type controls ($Casp11^{Vda+/+}$, n = 6). Error bars of body temperature represent SEM. $^{**}p < 0.01$. Log rank (Mantel-Cox) test (C and D) and F-test (E). See also Figure S2 and Table S3.

sepsis (Vandenbroucke and Libert, 2014; Vanlaere and Libert, 2009).

Opposing Phenotypes in 129 and C57BL/6 ESC-Derived *Panx1* Null Mice after Lethal LPS Challenge

In addition to several MMP family members, the Pannexin 1 (*Panx1*) locus resides within a 4 cM distance from the *Casp11* locus (Table S3). Conceptually, one could study the LPS susceptibility of *Panx1* null mice considering the following data: the molecular interplay between the hemichannel Pannexin 1 and the *Nlrp3* inflammasome, the proposed role of Pannexin 1 in delivery of LPS to the cytosol (Kanneganti et al., 2007), and the LPS-resistance of *Nlrp3* null mice (Mariathasan et al., 2006). Indeed, *Panx1* null mice generated using 129-derived ESCs (backcrossed to C57BL/6 for 5 generations) originating from V.I.S.'s lab ($Panx1^{Vsh}$ null mice) were resistant to death (Figure 3C) and hypothermia (Figure 3D) caused by *E. coli* LPS. The resistance to LPS was again similar as observed in *Casp11* null mice (Figures 3G and 3H). Importantly, $Panx1^{Vsh}$ null mice were also affected by the *Casp11* passenger mutation (Figures 3A and 3B). In contrast, *Panx1* null mice generated in V. M. Dixit's lab from C57BL/6-derived ESCs ($Panx1^{Vmd}$ null mice) were not protected against death (Figure 3E) and hypothermia (Figure 3F) caused by LPS, which is associated with the presence of a WT allele for *Casp11* (Figures 3A and 3B). In conclusion, our analysis of different *Mmp*- and *Panx1* null mouse lines illustrates how the presence or absence of a phenotype (protection against lethal endotoxemia) coincides with the presence or absence of one passenger mutation, in casu mutant *Casp11* derived from the 129 genome, and can produce "false-positive" phenotypes.

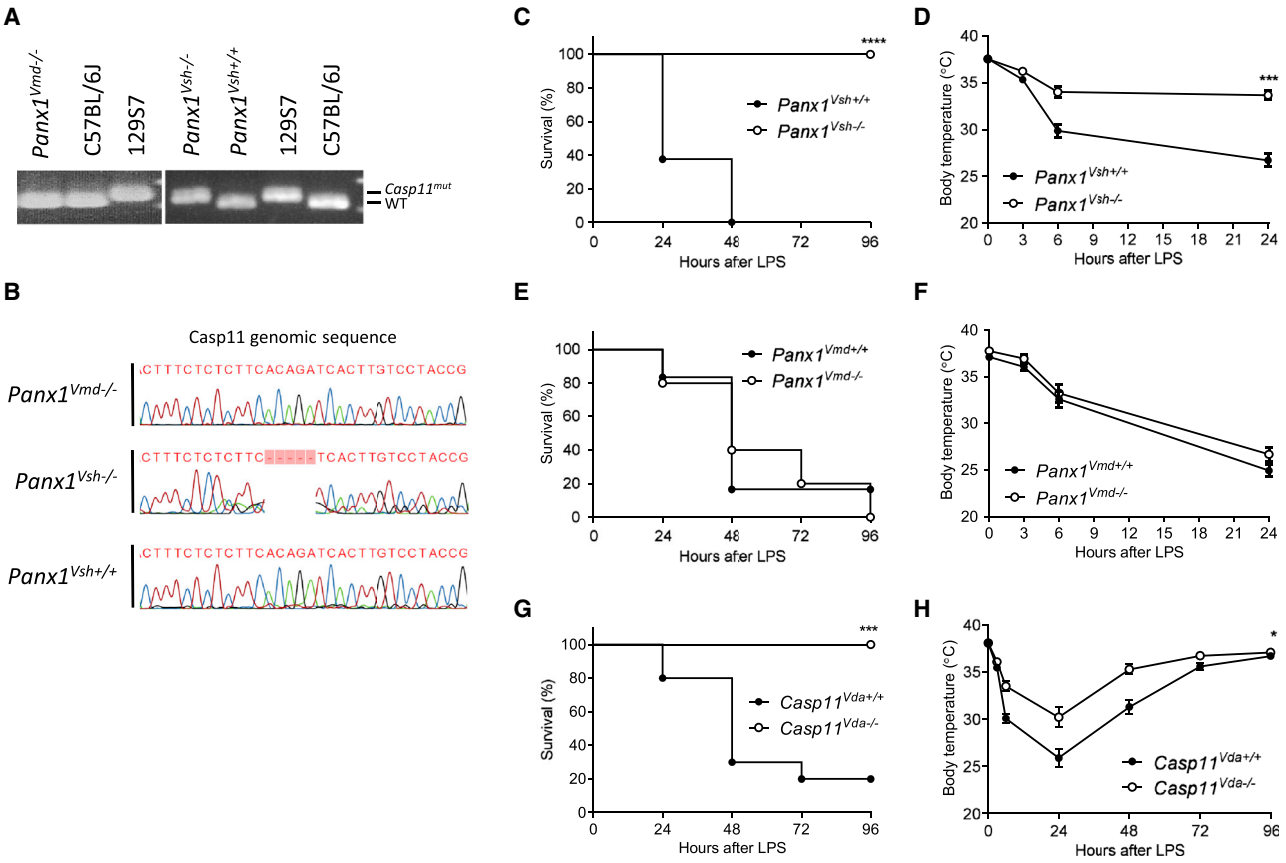

Figure 3. Presence of *Casp11* Inactivating Passenger Mutation in 129 ESC-Derived *Panx1* Null Mice, but Not in C57BL/6 ESC-Derived *Panx1*-Null Mice, Results in Resistance to LPS-Induced Shock

(A) Gel electrophoresis-separated PCR fragments of the genomic DNA region flanking the 5-bp deletion in *Casp11* gene in Panx1 null mice.

(B) DNA sequencing profiles of the genomic DNA region flanking the 5-bp deletion in *Casp11* gene in Panx1 null mice. C57BL/6 and 129 strains were used as negative and positive controls, respectively, for the 5-bp deletion in *Casp11*.

(C and D) Survival and body temperature were analyzed in function of time after intraperitoneal injection of 15 mg kg^{-1} *E. coli* LPS in *Panx1* null mice derived from V.I.S.'s lab (*Panx1*$^{Vsh-/-}$, n = 10), which carry the *Casp11* passenger mutation, and matching wild-type controls (*Panx1*$^{Vsh+/+}$, n = 8).

(E and F) Survival and body temperature were analyzed in function of time after intraperitoneal injection of 15 mg kg^{-1} *E. coli* LPS in Panx1 null mice derived from V.M. Dixit's lab (*Panx1*$^{Vmd-/-}$, n = 5), which have a wild-type allele for *Casp11*, and matching wild-type controls (*Panx1*$^{Vmd+/+}$, n = 5).)

(G and H) Survival and body temperature were analyzed in function of time after intraperitoneal injection of 15 mg kg^{-1} *E. coli* LPS in *Casp11* null mice (*Casp11*$^{Vda-/-}$, n = 8) and matching wild-type controls (*Casp11*$^{Vda+/+}$, n = 10). Survival was analyzed using Log rank (Mantel-Cox) test. Body temperature curves were analyzed using F-test. *p < 0.05, ***p < 0.001, ****p < 0.0001. Error bars of body temperature represent SEM. See also Table S3.

Validation of Two Predicted Protein Coding Variants in 129 Strain

Next, we selected one 129-derived indel (predicted as a "frameshift variant") in a gene involved in metabolism, viz. aldehyde oxidase 4 (*Aox4*) and one SNP (predicted as a "gained STOP") in a gene involved in immune regulation viz. serine peptidase inhibitor, clade A, member 3I (*SerpinA3i*). We confirmed the predicted gained premature STOP codon in *SerpinA3i* (Figures S3A and S3B) and T nucleotide insertion in *Aox4* (Figure S4A), which also results in a STOP codon after 11 codons (Figure S4B). For a flanking region of 10 cM, the 129-derived *SerpinA3i* and *Aox4* passenger mutations could affect, respectively, 101 and 120 of the currently available 129-derived genetically modified congenic mice (Figures S3C and S4C). For example, the *Casp8* gene is ≤1cM distance from *Aox4*. We confirmed that the commonly used 129-derived *Casp8* conditionally deleted congenic mouse line (*Casp8*$^{Hed\_FL/FL}$) contains this *Aox4* inactivating mutation, which is absent in C57BL/6JN-derived *Casp8*$^{Tvb\_FL/FL}$ mice (Figure S4A). These two validated case studies underscore the strength of the genomic sequence released by Sanger Institute (Keane et al., 2011) and the associated protein-coding variants predictions.

C57BL/6J and C57BL/6NJ Sub Strains Exhibit Opposing Phenotypes after Lethal LPS Challenge

The International Mouse Phenotyping Consortium (IMPC) was founded to avoid the effects of genetic background variation in transgene technology (Austin et al., 2004; Skarnes et al., 2011). The consortium intends to mutate all protein-coding genes in the mouse using a combination of gene trapping and gene targeting in C57BL/6 mouse ESCs instead of the previously used 129 ESCs. To date, this initiative has resulted in 29% of all

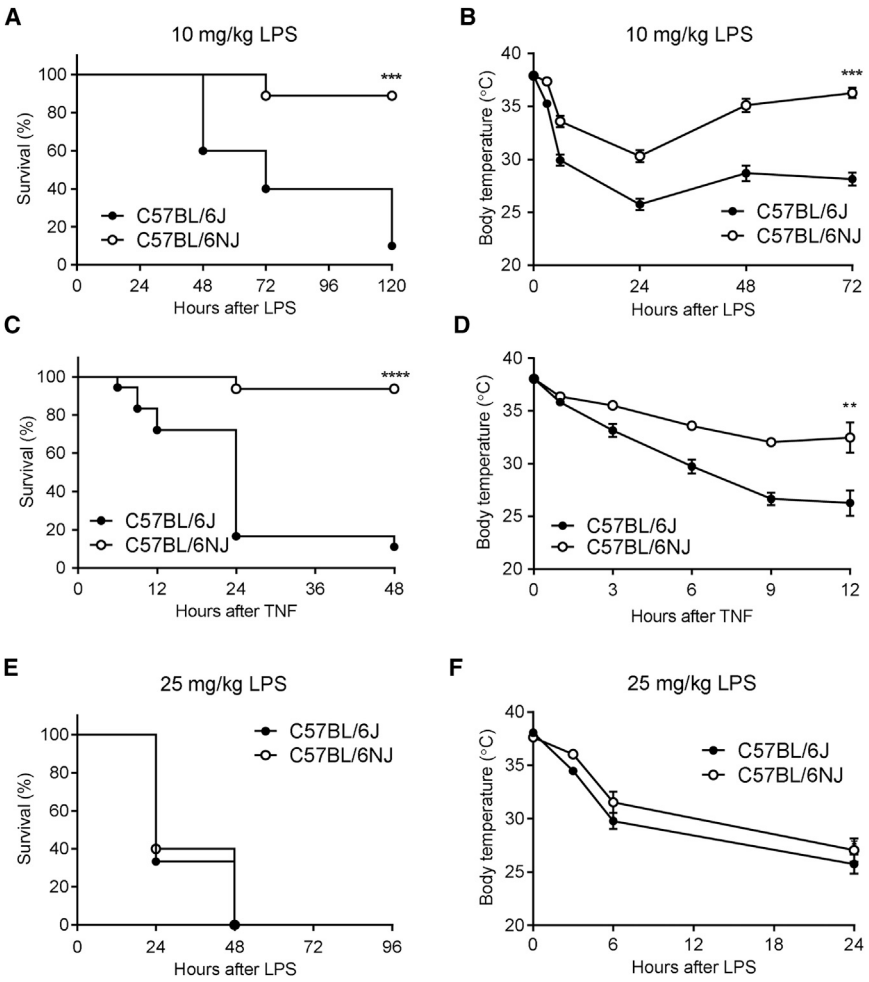

Figure 4. Susceptibility of C57BL/6 Substrains J and NJ Show Significant Difference to LPS- and TNF-Induced Lethal Shock

(A and B) Survival and body temperature were analyzed in a function of time after intraperitoneal injection of 10 mg kg^{-1} *E. coli* LPS in C57BL/6J (n = 10) and C57BL/6NJ mice (n = 9). The combined results of two independent experiments are shown.

(C and D) Survival and body temperature were analyzed in function of time after intravenous injection of 500 μg kg^{-1} TNF in C57BL/6J (n = 18) and C57BL/6NJ mice (n = 16). The combined results of two independent experiments are shown.

(E and F) Survival and body temperature were analyzed in function of time after intraperitoneal injection of 25 mg kg^{-1} *E. coli* LPS in C57BL/6J (n = 9) and C57BL/6NJ mice (n = 5). Error bars of body temperature represent SEM. **p < 0.01, ***p < 0.001, ****p < 0.0001. Log rank (Mantel–Cox) test (B and D) and F-test (A and C). See also Table S4.

genetically modified mice that originate from IMPC (Figure 1D). Importantly, the consortium uses ESCs derived from the C57BL/6NJ mouse, which could be a problem because it was separated from the commonly used inbred C57BL/6J strain 63 years ago. Indeed comparison of the genomes of these strains (Mouse Genomes project [Keane et al., 2011]) revealed 34 SNPs and two indels (Simon et al., 2013). We confirmed these two previously reported indels (Simon et al., 2013). However, we predict 28 additional indels and 6 SNPs that result in an alternative or aberrant amino acid sequence (Table S4). To phenotypically compare the strains in the context of lethal shock, we rederived both C57BL/6NJ and C57BL/6J in our specific pathogen free (SPF) facility by embryo transfer to circumvent pathogenic background effects. In line with the previously reported phenotypic differences between C57BL/6J and –NJ (Simon et al., 2013), we found that C57BL/6NJ mice were significantly more resistant to lethal shock induced by LPS or TNF, as shown by the survival rates (Figures 4A and 4C) and body temperature (Figures 4B and 4D), indicating that even the small genetic difference between the two C57Bl/6 strains is sufficient to generate phenotypic differences in these experimental disease models. Notably, this phenotypic difference between C57BL/6 sub strains in survival (Figure 4E) and hypothermia (Figure 4F) faded out by using a supralethal dose of LPS (2.5 × LD^{100}).

Web Tool to Identify Passenger Mutation in Genetically Modified Congenic Mice

To allow researchers to estimate the impact of passenger mutations on their particular genetically modified congenic mice, we developed a passenger mutation informatics search tool dubbed "MePaMuFind-It" (http://me-pamufind-it.org) that can easily verify the predicted identity and number of passenger mutations in 129-derived genetically modified congenic mice. The tool generates the following data sets: (1) a list of potential passenger mutations in the transgenic mouse of interest according to the number of backcrosses, (2) the number and identity of indels and SNPs that result in alternative or aberrant protein expression (referred to as protein coding variants), (3) list of genes containing 129-derived untranslated region (UTR) and upstream variants that affect the entered gene, (4) direct access to PubMed reports on respective genes, (5) direct access to genetically modified mouse lines reported in Mouse Genome Informatics, and (6) a list of known genetically modified mouse lines affected by a particular validated 129-derived passenger mutation. This information permits to estimate the potential effect of particular passenger mutations on the phenotype of the transgenic mice of interest. Note that the functional effects of most predicted passenger mutations are still unknown.

DISCUSSION

The importance of genetic background in genetically modified congenic mice is generally well known. Typically, researchers use littermate controls to deal with this issue, instead of wild-type strain control (often C57BL/6 mice). However, this approach doesn't solve the issue of 129-derived passenger mutations present in the flanking regions of the target gene. In particular, the flanking region of the target gene will originate from the

acceptor (which is often C57BL/6 strain) in the wild-type littermate controls, while the region will originate from the donor (which was mostly 129-derived ESCs) in the genetically modified littermates. The situation is different in case of conditional gene modified mice. Because the target gene is flanked by two loxP or Frt sites in all littermates, all mice equally carry the same passenger mutations. Thus, the observed phenotypes in the Cre recombinase-expressing mice are likely due to the removal of the gene of interest. However, investigators should include extra control groups in their experimental models such as the particular mouse Cre line and/or Cre recombinase expressing heterozygote mice (gene$^{FL/+}$ *cre*$^{Tg/+}$) to exclude phenotypic interference from the mouse Cre line. Of note, it is still possible that the observed phenotype is influenced by the flanking 129-derived passenger mutations.

What can be done to avoid this "spill over" of passenger mutations? It is advised to construct transgenic mice on a pure genetic background so that the resulting mice are "isogenic" rather than congenic. Essentially, one should cross the obtained chimeras with mice with the same genetic background as the ESCs used to generate transgenic mice. Essentially, the IMPC was founded to avoid the effects of genetic background variation in transgene technology and intends to mutate all protein-coding genes in the mouse using a combination of gene trapping and gene targeting in C57BL/6NJ mouse ESCs instead of the previously used 129 ESCs (Austin et al., 2004; Skarnes et al., 2011). In addition, the EUCOMMTOOLS project is engineering 500 Cre C57Bl/6NJ ESC lines to enable researchers to use transgenic mice with a defined genetic background (Murray et al., 2012). Alternatively, multiplex genome engineering technologies such as CRISPR/Cas systems have also been widely introduced (Cong et al., 2013; Jinek et al., 2012; Mali et al., 2013; Platt et al., 2014), which will also help to circumvent problems associated with genetic background. Nevertheless, one has to be cautious about nonspecific integration often associated with CRISPR/Cas genome editing, which are currently minimized by using optimized adapted methods (Shen et al., 2014).

Our finding that nearly all 129-derived genetically modified congenic mice contain multiple passenger mutations despite intensive backcrossing implies that the phenotypes observed in these mice might be due to flanking passenger mutations rather than the defect in the targeted gene. Considering that almost 8,000 different genetically modified congenic mice are populated with 129-derived passenger mutations, it will be a challenge to elucidate the causative link between the targeted gene and the observed phenotypes. We exemplify this in the current study using the recently identified 129-derived *Casp11* passenger mutation, which potentially affects 86 genetically modified congenic mice (Tables S3 and S4). We show that 129-derived genetically modified congenic mice targeting some MMP family members and PANX1 are affected by this 129-derived *Casp11* inactivating mutation, which consequently provides these mice resistance to LPS. Basically, in case that the passenger mutation interferes with the experimental model, there is a risk of false positive phenotypes. Of the 1084 genes predicted to be aberrantly expressed in 129 mice, we selected and validated two protein coding variants involved in metabolism and immune regulation viz. *Aox4* and *SerpinA3i.* Considering that SERPINA3 inhibits cathepsin G released by activated neutrophils (Heit et al., 2013), one can expect phenotypic immunological inference of SERPINA3 inactivation in the 101 reported 129 ESC-derived genetically modified mouse lines of which the targeted gene flanks the *SerpinA3i* locus. In an attempt to link the *Aox4* passenger mutation to a phenotype observed in genetically modified congenic mice targeting an *Aox4*-neighboring gene, we found that mice deficient in phospholipase Cε (PLCε) showed a similar thickening of the epidermis (Martins et al., 2014) as observed in *Aox4* null mice (Terao et al., 2009). This epidermal thickening in the PLCε-deficient mice is potentially due to the 129-derived *Aox4* passenger mutation.

Several reports have identified 129-derived protein coding variants in for example DAP12 (*Tyrobp*) signaling (McVicar et al., 2002), DNA polymerase iota (*Poli*) (McDonald et al., 2003), beaded filament structural protein 2 (*Bfsp2*) (Alizadeh et al., 2004; Sandilands et al., 2004), disrupted-in-schizophrenia (*Disc1*) (Gómez-Sintes et al., 2014; Koike et al., 2006), apolipoprotein A-II (*Apoa2*) (Su et al., 2009), oculocutaneous albinism II (*Oca2*) (Rogers et al., 2012), and chromatin licensing and DNA replication factor 1 (*Cdt1*) (Coulombe et al., 2013). The 129-derived protein coding variant in *Bfsp2* and *Apoa2*, respectively, results in defects in the lens (Sandilands et al., 2004) and high density lipoprotein (HDL) metabolism (Su et al., 2009). Considering genetically modified congenic mice that target genes that flank ($\leq$10 cM) the two variants, we found genetically modified mouse lines reported with similar phenotypes that might be due to the passenger rather than the targeted gene itself. For example, in case of *Apoa2*, we found genetically modified mouse lines targeting 11β-Hydroxy-steroid dehydrogenase type 1, which show increased HDL amounts (Morton et al., 2001) and TLR5, which have increased adiposity (Vijay-Kumar et al., 2010). In case of *Bfsp2*, we found genetically modified mouse lines targeting gluthation peroxidase 1 (Spector et al., 1998; Spector et al., 1996) and αB-crystallin (Brady et al., 2001).

It is important that the scientific community becomes more aware of the broad impact of passenger mutations that affect phenotypes of mice in a wide variety of experimental disease models and fundamental biological studies. In summary, with 1,084 genes predicted to be aberrantly expressed in 129 mice, our case studies reveal a highly underestimated issue of false-positive results using genetically modified congenic mouse lines. Our comparative genomic analysis predicts that nearly all 129-derived genetically modified congenic mice are affected by multiple inactivating passenger mutations despite intensive backcrossing to C57BL/6 mice. This implies that the phenotypes of most of these congenic mouse lines, and cells derived of these mice, may be heavily influenced by inactivating passenger mutations in the flanking regions of the targeted genes. Consequently, phenotypic studies using primary mouse embryonic fibroblasts, bone-marrow-derived macrophages, or lymphocytes derived from 129-derived genetically modified congenic mice are equally affected by these passenger mutations. We believe that the variable translatability of the results of mouse studies to humans might be partially due to the phenotypic interference of passenger mutations (Osuchowski et al., 2014). The Me-PaMuFind-It web tool will help researchers to estimate the number and effect of passenger mutations in their transgenic mice and to decide whether to regenerate them in a defined genetic background, e.g., via IMPC or using genome engineering technologies such as CRISPR/Cas.

EXPERIMENTAL PROCEDURES

Bioinformatics and Comparative Genome Analyses

We analyzed the SNP data release v3 (REL-1303-SNPs_Indels-GRCm238) from the Mouse Genome Project available at Sanger Institute (ftp://ftp-mouse.sanger.ac.uk/REL-1303-SNPs_Indels-GRCm38/; Keane et al., 2011). The data in the indel vcf file (mgp.v3.indels.rsIDdbSNPv137.vcf) and SNP vcf file (mgp.v3.snps.rsIDdbSNPv137.vcf) were filtered to retrieve indels and SNPs present in at least one of the three 129 strains (129P2/OlaH, 129S1/SvIm and 129S5SvEvB) and affecting the protein coding sequence of the genes. These so-called protein coding variants are based on the following sequence ontology (SO) terms: stop_gained, stop_lost, inframe_insertion, inframe_deletion, frameshift_variant, splice_donor_variant, splice_acceptor_variant, and coding_sequence_variant. In total, 949 indels and 446 SNPs affecting 1,084 mouse genes were retained. We gathered chromosome and gene start and end positions for 1,084 genes covering 1,395 variations (949 indels + 446 SNPs). The Ensembl gene ID (ENSMUSG) was used to find the most upstream and downstream start and stop in all Ensembl transcripts (ENSMUST) for that gene and saved as gene start and end. Next these genome coordinates were used to search for flanking genes within 2, 10, and 20 Mbps both upstream and downstream. We then downloaded all mouse phenotypic allele data from the MGI resource (ftp://ftp.informatics.jax.org) and extracted the data of genetically modified mouse lines. Information on 5,322 genes (corresponding to 7,979 129-derived genetically modified mouse lines) was connected to genes with passenger mutations and affected genes. Additionally we filtered the data for 5 kb upstream, 5′ UTR and 3′ UTR indels and SNPs to identify putative regulatory variants. This analysis retrieved in total 673,023 regulatory variants, which are listed separately for a gene of interest. All data were stored in a MySQL database and can be queried using the publicly available web tool Me-PaMuFind-It.

Development of the Me-PaMuFind-It Web Tool

It is important for scientists to easily check whether a 129-derived transgenic mouse might carry passenger mutations. We developed the web tool "Me-PaMuFind-It" (http://me-pamufind-it.org), which generates a list of the potential passenger mutations present in any 129-derived transgenic mouse.

To start a search with Me-PaMuFind-It, the user enters a gene symbol or database accession number and chooses the number of backcrosses. The user will then be given three main types of information. The upper panel gives the user more information about the gene itself. With one click the user can access the NCBI Entrez Gene page, Ensembl Gene Page, all the PubMed articles about the gene, detailed information about protein coding variants and putative regulatory variants of the gene in the three 129 strains, and detailed information on the available genetically depleted mice.

Most important, the middle panel shows all the protein coding variants in flanking genes that can influence the gene of interest. To get a better overview of the distance between these flanking genes and the gene of interest, we incorporated a "Visualize" function. This function shows a genomic map of 10 cM upstream and downstream of the gene, but the user can also visualize the 2 cM and 20 cM region. The user can start a Me-PaMuFind-It search of every gene in this genomic map simply by clicking on the gene. As the genomic sequence is available for three 129 substrains (129P2/OlaH, 129S1/SvIm and 129S5SvEvB), we indicated for every variant, which 129 strain is affected. Furthermore, the user can analyze the variant sequence with a click. Finally, we also incorporated a link to UCSC to enable the user to analyze the variant sequence in more detail.

If the gene of interest carries any protein coding variant, the lower panel lists all the genes that might be affected by this variant. The distance between the affected genes and the gene of interest is again indicated by percentages. Every gene mentioned in the Me-PaMuFind-It tool indicates whether a genetically depleted mouse exists. Additionally, the user can get detailed information about the genetically depleted mouse, such as the genetic background of the genetically depleted or the name of the lab that generated it. Finally, there is also a link to the Mouse Genome Informatics website.

Mice

All the mice were bred under specific-pathogen-free (SPF) conditions, unless indicated differently. Mice were housed in temperature-controlled, air-conditioned facilities with subsequent 14 hr light and 10 hr dark cycles, and food and water ad libitum, and were used at the age of 8–12 weeks. Sex- and age-matched animals were used in all experiments, which were approved by the animal ethics committee of Ghent University.

129S1/SvImJ mice (002448) were purchased from The Jackson Laboratory. C57BL/6J. C57BL/6N mice were purchased from The Jackson Laboratory and re-derived in our SPF animal facility. *Panx1* null mice generated using 129-derived (MGI:5487761) and C57BL/6-derived ESCs (MGI:5013648) were kindly provided by the V.I.S. and Dixit labs, respectively. *Mmp8* (MGI:2681103), *Mmp13*^{Cli} (MGI:3521852) and *Mmp19* null mice (MGI:3505589) were kindly provided by Dr. Carloz Lopez-Otin (Universidad de Oviedo, Spain). *Mmp3* (MGI:2386262), *Mmp7* (MGI:1857932), *Mmp9* (MGI:1932294), and Mmp*13*^{Ebb} null mice (MGI:3521852) were kindly provided, respectively, by Dr. John S. Mudget (Merck Research Laboratories, USA), Dr. Carole L. Wilson (Medical University of South Carolina, USA), Dr. Steven D. Shapiro (Washington University School of Medicine, USA), and Dr. Edward B. Brown (University of Rochester, USA). The *Mmp13*^{Cli} and *Mmp13*^{Ebb} null mice (MGI:3521852) both originated from Stephen M. Krane's lab (Harvard Medical School and Massachusetts General Hospital, USA). *Mmp9* and *-19* null mice were bred in our conventional mouse facility. The mice that were imported from USA (*Mmp13*^{Ebb} null mice from Brown's lab, and *Panx1* null mice from V.I.S. and Dixit labs) had been bred under SPF conditions in the respective labs and transferred to our conventional mouse facility in individual ventilated cages for LPS lethality studies.

Mouse Genomic DNA and Genotyping

Mouse genomic DNA prepared from mouse tails, unless indicated differently, was donated by the following persons: *Mmp28* null mice, Dr. Anne Manicone, (University of Washington, USA); *Mmp9* null mice, Dr. Anna Rosell (Institut de Recerca Hospital Vall d'Hebron, Barcelona); Mmp14 null mice, Dr. Paola Zigrino (University of Cologne, Germany); *Mmp7* null mice, Dr. Sarah Jane George (School of Clinical Sciences, UK); *Mmp3* null mice, Dr. Ranjan Gupta (University of California, USA); *Mmp19* null mice, Dr. Annie Pardo (Ciudad Universitaria, Mexico); and *Mmp13*^{Ebb} null mice, Dr. Seth Perry (University of Rochester, USA). Genomic DNA was prepared locally from mouse tails of *Mmp8* null mice bred in house, and from frozen sperm of *Mmp13*^{Cli} null mice stored in house.

The *Casp11* inactivating passenger mutation was identified by PCR using primers 5′-AGGCATATCTATAATCCCTTCACTG-3′ and 5′-GGAATATATCAAAGAGATGACAAGAGC-3′. Temperature scheme standard PCR: 4 min 94°C, 1 min 94°C, 0.5 min 58°C, 1 min 72°C, 7 min 72°C, and end at 12°C. Samples were loaded on 3% agarose gel, and the 5 bp-deletion *Casp11* fragment runs slightly higher than the wild-type fragment at a high of approximately 220 bp. In addition, the 5-bp deletion (*Casp11* inactivation) was confirmed by sequencing a PCR product using primers 5′-CAGTATTATTATTGGTGATGCAAATG-3′ and 5′-GGAATATATCAAAGAGATGACAAGAGC-3′.

Reagents and Injections

Mice were injected intraperitoneally (i.p.) with the indicated dose of LPS from *Escherichia coli* O111:B4 (cat n°: L-2630, Sigma-Aldrich) or *Salmonella enterica* serotype abortus equi (cat n°: L-5886, Sigma-Aldrich) suspended in LPS-free PBS. Mice were injected intravenously (i.v.) with the indicated dose of TNF (purified in house) suspended in LPS-free PBS.

Statistics

All statistics were performed using Prism software (GraphPad Software) or Genstat (Payne et al. Genstat Release 16.1 Reference Manual, Part 3. VSN International). All experiments were set up as a completely randomized design. Survival curves were compared with a Mantel-Cox test. We used restricted maximum likelihood (REML) as implemented in Genstat (Payne et al. Genstat Release 16.1 Reference Manual, Part 3. VSN International) to perform the repeated-measurements analysis for body temperature. Repeated-measurements data were analyzed by fitting the following linear mixed model (random terms underlined): $y_{ijrt} = \mu + genotype_i + time_t + genotype.time_{it} + \underline{experiment_r} + \underline{subject_{ijr}} + \underline{subject.time_{ijrt}}$, where y_{ijrt} is the body temperature of the *j*th individual of genotype *i*, measured in the *r*th experiment at time point *t*. The random term *subject.time* was used as residual term with correlation structure. Various ways of modeling the correlation structure (unstructured, antedependence

order 1 and 2, power model [city block metric]) were compared in the residual maximum likelihood (REML) framework, with time points set as not equally spaced. Selection of the best model fit was based on a likelihood ratio test (LRT) statistic and/or the Aikake Information coefficient (AIC). When residuals from the analysis indicated increasing variance over time, this was modeled directly by specifying that heterogeneity is to be introduced into the model. Significance of the fixed main and interaction effects was assessed by an *F* test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2015.06.011.

AUTHOR CONTRIBUTIONS

T.V.B. and P.V. designed the research and wrote the paper. T.V.B. carried out experiments and analyzed the data. C.L. assisted with research design, data interpretation, and writing of the paper. P.H. and L.M. performed bioinformatic analyses and developed the Me-PaMuFind-It web tool. R.E.V., I.B., T.D., E.V.W. and S.M.C carried out experiments. S.W.P. and V.I.S provided Mmp13- and Panx1-null mice, respectively, and assisted in the writing of the paper. M.V. performed statistics.

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