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Genome-wide Generation and Systematic Phenotyping of Knockout Mice Reveals New Roles for Many Genes

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SUMMARY

Mutations in whole organisms are powerful ways of interrogating gene function in a realistic context. We describe a program, the Sanger Institute Mouse Genetics Project, that provides a step toward the aim of knocking out all genes and screening each line for a broad range of traits. We found that hitherto unpublished genes were as likely to reveal phenotypes as known genes, suggesting that novel genes represent a rich resource for investigating the molecular basis of disease. We found many unexpected phenotypes detected only because we screened for them, emphasizing the value of screening all mutants for a wide range of traits. Haploinsufficiency and pleiotropy were both surprisingly common. Forty-two percent of genes were essential for viability, and these were less likely to have a paralog and more likely to contribute to a protein complex than other genes. Phenotypic data and more than 900 mutants are openly available for further analysis.

INTRODUCTION

The availability of well-annotated genome sequences for a variety of organisms has provided a strong foundation on which much biological knowledge has been assembled, including the generation of comprehensive genetic resources. This has been

achieved in several model organisms, including *E. coli*, *S. cerevisiae*, *S. pombe*, *A. thaliana*, *C. elegans*, and *D. melanogaster*, greatly facilitating studies focused on single genes and enabling genome-wide genetic screens.

Annotation of the human genome has identified over 20,000 protein-coding genes as well as many noncoding RNAs. Despite the dramatic increase in the knowledge of variation in human genomes, the normal function of many genes is still unknown or predicted from sequence analysis alone, and consequently, the disease significance of rare variants remains obscure. Furthermore, there remains a large bias toward research on a small number of the best-known genes (Edwards et al., 2011). Realizing the full value of the complete human genome sequence requires broadening this focus, and the availability of comprehensive biological resources will facilitate this process.

The mouse is a key model organism for assessing mammalian gene function, providing access to conserved processes such as development, metabolism, and physiology. Genetic studies in mice, mostly via targeted mutagenesis in ES cells, have described a function for 7,229 genes (ftp://ftp.informatics.jax.org/pub/reports/MGI_PhenotypicAllele.rpt, February 2013). The vast majority of these studies have been directed at previously studied (known) genes, driven by previous biological knowledge. Phenotype-driven screens have also identified genes associated with specific phenotypes, although to a smaller extent. Although targeted mutagenesis has been very successful, the global distribution of the effort has resulted in significant heterogeneity in allele design, genetic background of mice used, and their phenotypic analysis. Furthermore, the biological focus of most targeted knockout experiments is constrained by the expertise of the

specific research group. As a result, many phenotypes have not been detected, and consequently, the full biological function of many genes studied using knockout mice is significantly underreported.

Some efforts to generate and phenotype sizeable sets of new targeted alleles of genes of interest have been reported previously (e.g., Tang et al., 2010). These studies focused on specific categories of molecules such as secreted and transmembrane proteins or other “drugable” targets. Other research centers have established mouse clinics, with the aim of carrying out a comprehensive analysis of the phenotypes of mutant lines of specific interest (e.g., Fuchs et al., 2012; Wakana et al., 2009; Laughlin et al., 2012).

The genome-wide set of targeted mutations in ES cells established by the KOMP, EUCOMM, and MirKO programs (Skarnes et al., 2011; Prosser et al., 2011; Park et al., 2012) provides an opportunity to conduct systematic, large-scale gene function analysis in a mammalian system without the variables inherent in studies by individual groups. The Sanger Institute’s Mouse Genetics Project (MGP) was one of the first programs to pursue this objective, established in 2006 when the first targeted ES cells became available. The MGP later expanded to contribute to a European phenotyping effort, EUMODIC, and more recently has become a founding member of the International Mouse Phenotyping Consortium (IMPC). Summaries of the developing efforts and aspirations of the IMPC have been reported (e.g., Brown and Moore, 2012; Ayadi et al., 2012). As the first established large-scale project using the KOMP/EUCOMM ES cells, the MGP has provided pilot data to inform the design of the international effort, such as the advantages of a single pipeline design, optimum numbers of mice, and details of variance for specific phenotyping tests. To date, the MGP has generated more than 900 lines of mutants using KOMP/EUCOMM resources (<http://www.sanger.ac.uk/mouseportal/>), and here, we describe the analysis of 489 of these for viability and fertility and 250 lines that have passed through a systematic screen for adult phenotypes, providing a glimpse into the wealth of biological insight that will emerge from these programs. Publicly available data enable the construction of new hypotheses, and the mouse mutants provide an invaluable resource for follow-up studies.

RESULTS

Genes and Alleles

Mice carrying targeted knockout first conditional-ready alleles from the KOMP/EUCOMM ES cell resources (Figures S1A and S1B available online; Skarnes et al., 2011) were established on a C57BL/6 genetic background. The mutants generated are listed in Tables S1 and S2, and all are available through public repositories including EMMA (<http://www.emmanet.org/>) and KOMP (<http://www.komp.org/>). Two classes of alleles are represented: those targeted with a promoter-driven selectable marker, and those with promoterless targeting vectors. Most are expected to be null alleles based on previous experience with this design (Mitchell et al., 2001; Testa et al., 2004). Data from 25 alleles showed that most (15) had <0.5% of normal transcript level detected in liver with a minority (4) showing a “leaki-

ness” of ~20% (column X; Table S2). The structure of each allele was confirmed when established in mice (Figure S1C).

Viability

Viability was assessed at postnatal day 14 (P14) by genotyping offspring of heterozygous crosses (Figure 1A). Data from 489 targeted alleles are summarized in Figure 2A. Overall, 58% were fully viable, whereas 29% produced no homozygotes at P14 and were classed as lethal, consistent with the proportion of homozygous embryonic/perinatal lethal mutants reported by MGI (2,183 of 7,229 lines of mice [30%]; ftp://ftp.informatics.jax.org/pub/reports/MGI_PhenoGenoMP.rpt, February 2013). A further 13% produced fewer than 13% homozygotes and were considered to be subviable. Genes required for survival included alleles generated with both promoter-driven and promoterless selection cassettes, but the latter were significantly more likely to be lethal (Figure 2B; Table S3) despite a greater level of persistent gene expression (11 of 14 promoter-driven compared with 4 of 11 promoterless alleles with <0.5% expression; column X; Table S2).

Alleles classed as lethal or subviable at P14 were further assessed at E14.5 (Figure 2C). From 143 alleles examined, 48% (68 genes) produced no homozygotes, indicating embryonic lethality and complete resorption by E14.5. One-third of alleles ($n = 45$) produced the expected number of homozygous embryos, whereas 30 (21%) produced fewer than expected homozygotes. Of the 75 mutant lines that produced homozygous embryos, 34 exhibited one or more morphological defect (Figure 2D). Some mutants ($n = 23$) presented with specific abnormalities including craniofacial defects and polydactyly, whereas 11 lines displayed only generalized indicators of developmental defects, edema, and/or growth retardation (Figure 2D). Examples are illustrated in Figures 2E–2G.

Fertility

Fertility of heterozygotes was assessed from heterozygous intercrosses. Of 489 alleles assessed, all heterozygotes were able to produce offspring. Homozygous mutants for 307 of the viable lines were then assessed. A homozygous infertility rate of 5.2% ($n = 16$) was observed (Figure 2H), strongly male biased with 15 of 16 genes exhibiting male infertility. A total of 11 genes affected only males, whereas just 1 was female specific (*Pabpc1*). Of these 16 genes, 7 have not previously been associated with infertility. Although some were good candidates such as *Usp42*, expressed during mouse spermatogenesis (Kim et al., 2007), others are novel genes such as *3010026O09Rik* and may suggest new pathways or mechanisms influencing fertility.

Adult Phenotypes

We report here the results of our screen of the first 250 lines to complete all primary phenotyping pipelines. In contrast to previous focused screens by Mitchell et al. (2001) and Tang et al. (2010), a broad range of gene products was included. The 250 genes reported span all chromosomes except Y (Figure S1D) and include eight control lines published previously and 87 genes proposed by the research community. For 34 of the 250 genes, no functional information has been published. A comparison of this gene set with all mouse genes indicates minimal

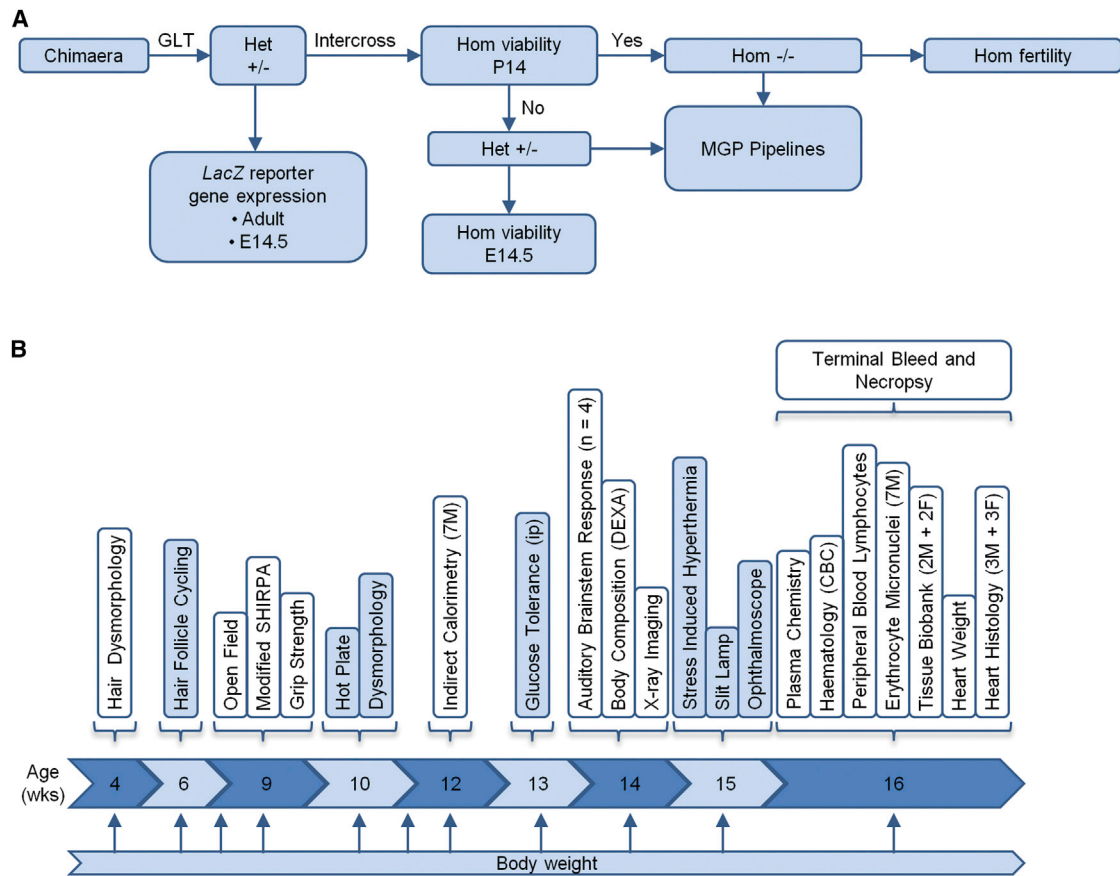


Figure 1. Illustration of the Phenotyping Pipelines

(A) An overview of the typical workflow from chimera to entry into phenotyping pipelines, encompassing homozygous (Hom) viability, fertility, and target gene expression profiling using the *lacZ* reporter. Het, heterozygous.

(B) The Sanger Institute MGP clinical phenotyping pipeline showing tests performed during each week. Seven male and seven female mutant mice are processed for each allele screened. In addition, seven male and seven female WT controls per genetic background are processed every week. See also [Figure S1](#) and [Tables S1](#) and [S2](#).

GO term enrichment spread over a variety of processes and underrepresentation only in sensory perception of smell, indicating that the gene set can be regarded as a reasonable sample of the genome.

A series of tests was used ([Figure 1B](#)), designed to detect robust variations in phenotypes that were key indicators of a broad spectrum of disease categories. Of the 250 reported lines, 104 were either lethal or subviable; most of these were screened as heterozygotes ($n = 90$), and the remaining lines were screened as homozygotes and/or hemizygotes ($n = 160$). All mutant lines generated passed through all primary phenotypic screens. For most tests in the pipeline, seven males and seven females were used, tested in small batches so that the data for each genotype were gathered on different days ([Figure S2A](#)). Assays culminated in the collection of samples at 16 weeks of age ([Table S4](#)). The primary screen included a high-fat diet challenge to exacerbate any latent phenotypes. Separate pipelines included challenges with two infectious agents: *Salmonella* Typhimurium and *Citrobacter rodentium* ([Table S4](#)).

Phenotypic data from the first 250 mutant alleles through the adult pipelines are summarized in [Tables S1](#) and [S2](#), with signif-

icant differences from the control baseline (hits) indicated by a red box. To make robust phenotypic calls, a reference range method was implemented that uses accumulated wild-type (WT) data to identify and refine the 95% reference range ([Figure S2B](#)). Mutant data were compared to the relevant reference range and variant phenotypes determined using a standardized set of rules ([Figure S3](#)). We aimed to highlight phenotypes with large effect sizes. This approach results in conservative calls and minimizes false positives. There was very little missing data (2.14% of all calls; [Table S2](#)). The maximum number of parameters collected per line was 263. Of these, 147 were categorical variables, for example normal or abnormal teeth, whereas 116, such as plasma magnesium levels, exhibited a continuous distribution from which outliers were identified. Examples of parameters with continuous variables (cholesterol, high-density lipoprotein [HDL], low-density lipoprotein [LDL], mean weights, and auditory brainstem responses [ABR]) are illustrated in [Figure 3](#).

Gene expression was examined by whole-mount *lacZ* reporter gene expression in 41 tissues and organs of adults, typically using heterozygotes (≥ 6 weeks old; $n = 243$ lines; [Table S1](#)).

Ubiquitous expression was recorded for eight lines (3.3%) and complete absence of expression in nine lines (3.7%). Of the remaining lines, 168 (69.1%) showed expression in <20 of the tissues, suggesting a relatively specific expression pattern, whereas 58 (23.9%) were more broadly expressed (≥ 20 tissues with detectable *lacZ* expression).

The data and images can be viewed on the Sanger Institute's mouse portal, accompanied by step-by-step examples of how to access the data (<http://www.sanger.ac.uk/mouseportal/>). Much of the raw data can be downloaded from the MGP Phenotyping Biomart (<http://www.sanger.ac.uk/htgt/biomart/martview/>) for further analysis. Summaries can be found by searching for each gene of interest in Wikipedia (http://en.wikipedia.org/wiki/Category:Genes_mutated_in_mice) and Mouse Genome Informatics (<http://www.informatics.jax.org/>).

Many Unexpected Phenotypes Discovered

A few examples of the wide range of phenotypes we discovered are illustrated in Figure 4. Body weight and fat/lean composition were among the most common anomalies, with both overweight ($n = 2$) and underweight ($n = 21$) mutants discovered. The *Kptn* mutant is an example of an unexpected phenotype. *Kptn* is a putative actin binding protein proposed as a candidate for deafness because it is expressed in sensory hair cells (Bearer et al., 2000). Instead, the homozygous *Kptn* mutant has increased body weight on a high-fat diet (Figure 4A) and increased bacterial counts following *Salmonella* Typhimurium challenge but normal hearing (Table S2). Additional new phenotypes were detected in genes that had been published previously, such as reduced grip strength and ankylosis of the metacarpophalangeal joints in *Dnase1l2* mutants (Fischer et al., 2011), delayed response in the hot plate test in *Git2* mutants (Schmalzigaug et al., 2009), and small sebaceous glands in *Cbx7* mutants (Forzati et al., 2012) (Figures 4B–4E, 4G, and 4H). Phenotypes were also detected in genes that had not been published previously, such as impaired hearing in *Fam107b* mutants and elevated plasma magnesium concentration in *Rg9mtd2* mutants (Figures 4F and 4I, respectively). These examples demonstrate that many phenotypes will be missed unless they are specifically looked for and illustrate the value of carrying out a broad range of screens with all mutants going through all screens. They also reveal our collective inability to predict phenotypes based on sequence or expression pattern alone.

Haploinsufficient and Nonessential Genes

Haploinsufficient phenotypes were detected in 38 of 90 (42%) of these lines. Thus, haploinsufficiency is relatively common, suggesting that screening heterozygotes or knockout lines can yield valuable insight into gene function and provide models for dominantly inherited human disorders. All 90 genes screened as heterozygotes had at least 1 hit (usually viability) and together gave a total of 181 hits (ranging from 1 to 14 per line), an average of 2.0 per line, or 1.0 per line if we consider that abnormal viability is a feature of the homozygote. The distributions of phenotypic hits are shown in Figures 5A and 5B. Two examples of haploinsufficiency are illustrated in Figures 4J–4N.

A total of 837 phenotypic variants were detected in the 250 mutant lines, 1.27% of the total calls (Tables S1 and S2). Of

the lines screened as homozygotes or hemizygotes, 35% (56 of 160) appeared completely normal in our screen. There are several possible explanations for the lack of a detected phenotype, such as incomplete inactivation of the gene, a subtle change in phenotype not detected by our screen, or the gene may be nonessential. So far, there is no overlap between the 56 mouse lines with no detected phenotype and genes homozygously inactivated in humans, but both data sets are limited in coverage to date (MacArthur et al., 2012). The remaining 104 homozygous/hemizygous lines gave a total of 656 hits (range 0–41 per line), an average of 6.3 hits per line.

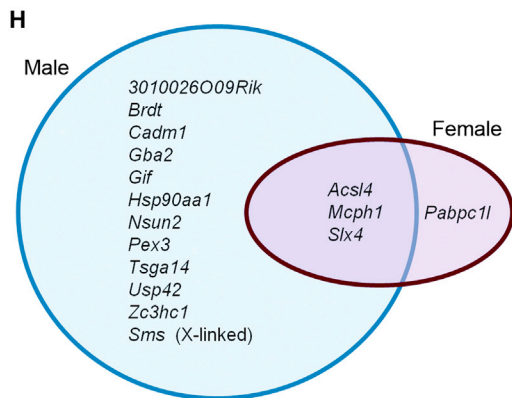
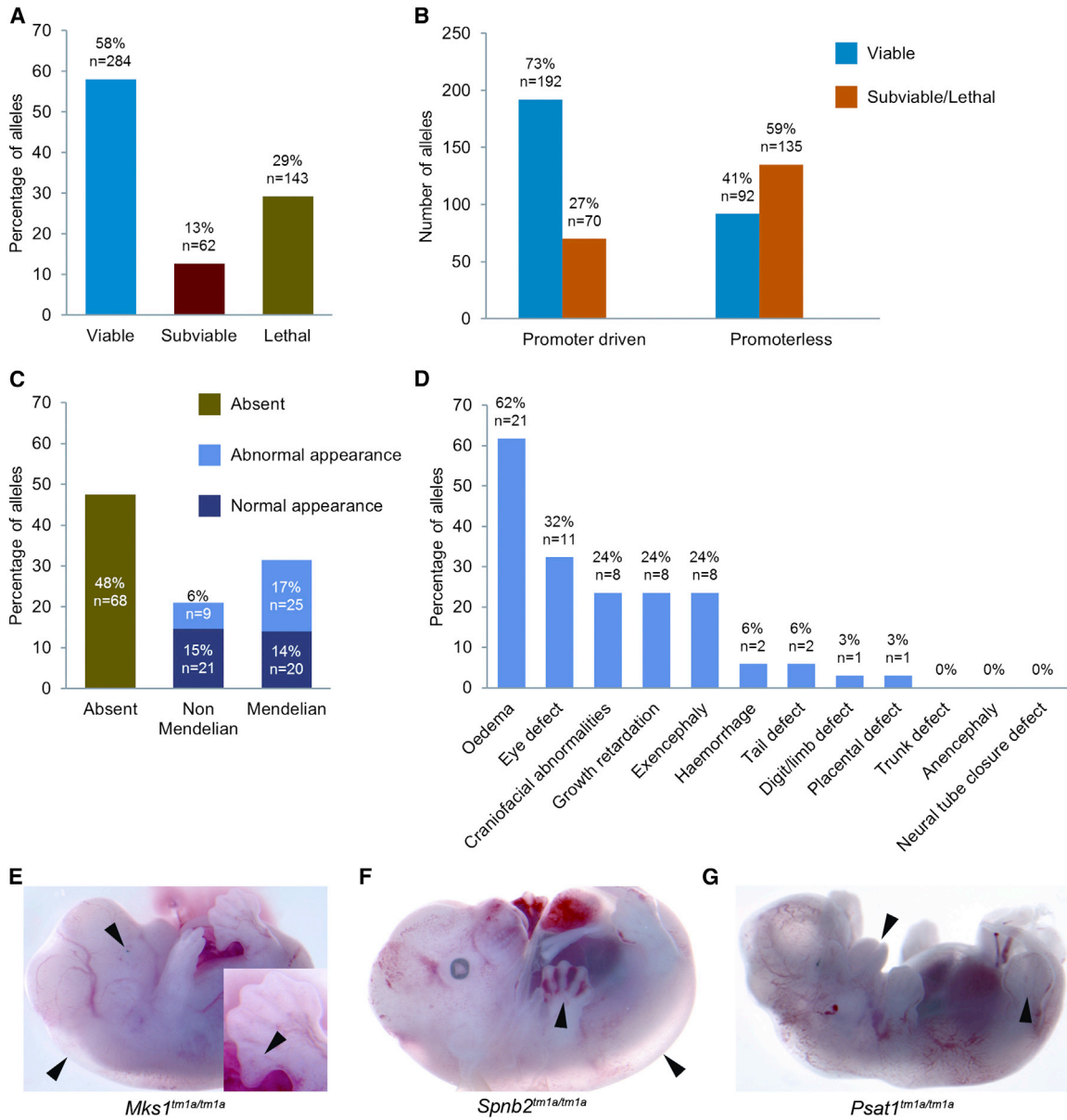
Sensitivity of the MGP Screen

To assess the sensitivity of our screen, the phenotypes were compared with published data on alternative alleles where available. A total of 91 of 250 genes had published data reported in MGI (Table S5), and for 61 of these, our observations detected features of the published phenotypes. Importantly, for 56 genes, a new phenotype was detected by our screen (column K, Table S5). For 31 genes, features of the published phenotype were assessed but not detected by our pipeline. For example, *Asxl1^{tm1Bc/tm1Bc}* mice are published as being viable (Fisher et al., 2010), but we found that *Asxl1^{tm1a(EUCOMM)Wtsi}* homozygotes were lethal, with none detected among 276 progeny from heterozygous intercrosses ($\chi^2 = 95.13$, $df = 2$; $p < 2.2 \times 10^{-16}$). These discrepant cases may reflect differences in the allele and/or genetic background. In other cases (77 genes), the reported characteristics required a specialized test not included in our screen, such as the calcium signaling defect in cardiomyocytes of *Anxa6^{tm1Moss/tm1Moss}* mutants (Hawkins et al., 1999).

New Mouse Models for Human Disease

The data set reported here includes 59 orthologs of known human disease genes. We compared our data with human disease features described in OMIM (Table S6). Approximately half (27) of these mutants exhibited phenotypes that were broadly consistent with the human phenotype. However, many additional phenotypes were detected in the mouse mutants suggesting additional features that might also occur in patients that have hitherto not been reported. Interestingly, a large proportion of genes underlying recessive disorders in humans are homozygous lethal in mice (17 of 37 genes), possibly because the human mutations are not as disruptive as the mouse alleles. Of the 59 genes, 26 represent the first mouse mutant with publicly available data. Three examples (*Sms*, *Ap4e1*, and *Smc3*) representing the first targeted mouse mutant for each gene are illustrated in Figure 6, and all show similar phenotypic features to their human counterparts.

SMS mutations in humans cause X-linked Snyder-Robinson syndrome involving hypotonia, unsteady gait, diminished muscle mass, kyphoscoliosis, osteoporosis, facial asymmetry, and intellectual disability (Cason et al., 2003). Hemizygous *Sms* mutant male mice showed reduced muscle strength, lean mass and bone mineral density, lumbar lordosis (Figures 6A–6E), and growth retardation, recapitulating features of the human disease. In addition, male infertility was detected, suggesting a feature



(legend on next page)

that may not have been recognized in humans with *SMS* mutations.

Spastic paraplegia 51, autosomal recessive, is caused by mutations in *AP4E1* and leads to spastic tetraplegia with hyperreflexia and generalized hypertonia, microcephaly, intellectual disability and dilated ventricles, cerebellar atrophy, and/or abnormal white matter (Abou Jamra et al., 2011; Moreno-DeLuca et al., 2011). Homozygous *Ap4e1* mutant mice displayed several similarities such as increased lateral ventricle area, decreased corpus callosum span, and decreased rearing (Figures 6F–6J). In addition, hematological changes suggestive of anemia were detected in female *Ap4e1* mutants, which have not been reported in humans.

The third example is *SMC3*, associated with dominantly inherited Cornelia de Lange syndrome 3, featuring facial dysmorphism, hirsutism, growth retardation, neurodevelopmental delay, and upper-limb anomalies (Deardorff et al., 2007). *Smc3* mutant mice displayed homozygous lethality (prior to E14.5) and reduced heterozygote viability at P14 (45% instead of the expected 67%; $p' = 0.0064$). Surviving *Smc3* heterozygotes showed reduced body weight, and a subset showed a distinct craniofacial morphology (Figures 6K–6M). Distinct *Smc3* expression in hair follicles and key brain substructures was revealed using *lacZ* (Figures 6N and 6O), noteworthy because of the hirsutism and neurodevelopmental delay aspects of Cornelia de Lange syndrome 3. In addition, an increase in the number of helper and cytotoxic T cells was observed in the mutant mice, again indicating an aspect that might contribute to the phenotype of patients but that has not yet been reported.

Pleiotropic Effects of Mutations

The phenotypes detected in this study vary from discrete specific defects (e.g., decreased platelet cell number in *Crtf3^{tm1a}/tm1a* mutants) to complex phenotypes in which many organ systems are involved (e.g., *Spns2^{tm1a(KOMP)Wtsi}* homozygotes show eye, hearing, and immune defects; Nijnik et al., 2012). The distribution of phenotypic hits is shown in Figures 5A and 5B for homozygous and heterozygous mutants, respectively. The peak for homozygotes was the category with no detected abnormalities, whereas the second biggest group consists of mutants with just one phenotypic call. The lines examined as heterozy-

gotes all have at least one hit (viability), but 20 lines have in addition one other abnormal phenotype, and a handful have several. Classifying parameters into five disease categories, we analyzed the distribution of disease areas represented across all 250 mouse lines. The most common phenotypic call was in the category reproduction, development, and musculoskeletal (Figure 5C).

Some abnormal phenotypes are clearly not primary effects; for example, reduced weight may be a secondary consequence of a number of different primary defects. Given that certain phenotypic features would be expected to co-occur frequently, reflecting physiological or developmental associations, a principal component analysis was conducted to look for correlated patterns in the data. Plotting principal component 1 against 2 revealed four main clusters of mouse lines (colored ovoids in Figure 5D). The separation along principal component 2 arises from viability. The remaining separation of clusters marked by red and green from clusters marked blue and yellow (Figure 5D) arises from body weight and associated variables, including DEXA measurements and energy use (Figure 5E). Body weight is a common covariable in disease (Reed et al., 2008), so it is not surprising that it dominates the principal component analysis.

Features of Essential Genes

Genes are generally defined as essential if they are required for survival or fertility. Studies in yeast and worms suggest that genes with paralogs are much less likely to be essential, presumably because the paralog can compensate for the function of the inactivated gene (Gu et al., 2003; Conant and Wagner, 2004). Previous analyses of published data on mouse knockouts did not find a significant difference in essential genes between singleton and duplicated genes (Liang and Li, 2007; Liao and Zhang, 2007). However, the published gene set is biased toward genes involved in development (Makino et al., 2009). In contrast, we found that genes in our set without a paralog were more than twice as likely to be essential, a significant effect (Table S3; Figure 7A).

We next asked if the essential genes in our gene set are more likely to be involved in a protein complex, using an experimentally validated data set of human protein complexes from the

Figure 2. Homozygous Viability and Fertility Overview

(A) Homozygous viability at P14 was assessed in 489 EUCOMM/KOMP targeted alleles. A minimum of 28 live progeny were required to assign viability status. Lines with 0% homozygotes were classed as lethal, >0% and ≤13% as subviable, and >13% as viable.

(B) Comparison of homozygous viability data from targeted alleles carrying either a promoter-driven or promoterless neomycin selection cassette.

(C) Lines classed as lethal or subviable at P14 were further assessed for viability at E14.5. Of the 205 targeted alleles eligible for this recessive lethality screen, 143 are reported here. A total of 28 embryos were required to assign viability status, and outcomes were categorized by both the number and dysmorphism of homozygous offspring.

(D) A basic dysmorphism screen encompassing 12 parameters was performed on all embryos for the 75 targeted alleles classed as viable or subviable at E14.5. A total of 34 targeted alleles showed one or more abnormality, and the percentage incidence is presented.

(E–G) Examples of E14.5 dysmorphism (arrowheads indicate abnormalities) are presented. Homozygous progeny were detected at a Mendelian frequency in all three examples. Sixty-seven percent (six of nine) *Mks1^{tm1a/tm1a}* embryos presented with edema, polydactyly, and eye defects (E). Sixty-two percent (five of eight) *Spnb2^{tm1a/tm1a}* embryos presented with edema and hemorrhage (F). Eighty-six percent (six of seven) *Psat1^{tm1a/tm1a}* embryos presented with growth retardation, exencephaly, and craniofacial abnormalities (G).

(H) Fertility was assessed in homozygous viable lines (307 mouse lines assessed from a total of 331 eligible lines). At least four independent 6-week-old mice of each sex were mated for a minimum of 6 weeks, and if progeny were born, the line was classed as fertile, regardless of if the progeny survived to weaning. Of note is the strong skew toward male (blue circle) fertility issues (15 of 16 genes) compared to 4 of 15 genes that displayed female (red circle) fertility issues.

See also Table S3.

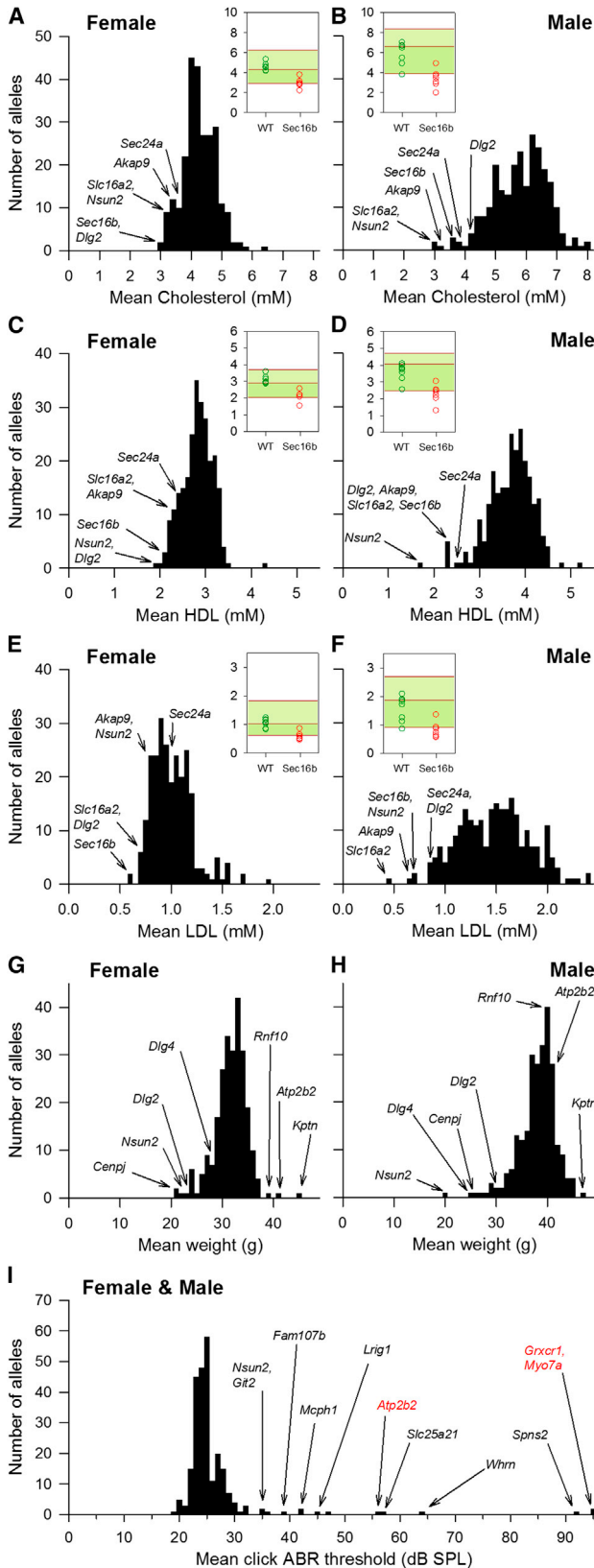


Figure 3. Data Distributions for Selected Parameters

(A–F) Distribution of mean total cholesterol (A and B), mean HDL cholesterol (C and D), and mean LDL cholesterol (E and F) at 16 weeks of age in both sexes for 250 unique alleles. Outliers are identified by gene name. The insets in (A)–(F) present the data for one outlier, *Sec16b^{tm1a/tm1a}* (red circles represent individual mice), compared to the WT controls processed during the same week (green circles), and a cumulative baseline of all WT mice of that age, sex, and genetic background (>260 WT mice) is presented as the median and 95% confidence interval.

(G and H) Distribution of mean body weight at 16 weeks in (G) female and (H) male mutant lines of mice. Outliers are identified by gene name.

(I) Distribution of mean click ABR threshold at 14 weeks (typically n = 4, independent of sex). Outliers are identified by gene name including positive controls highlighted in red.

CORUM database (Ruepp et al., 2010). We found that genes with a human ortholog that is part of a complex were significantly more likely to be essential (Table S3; Figure 7B).

Finally, we asked if there were certain types of gene products that were more likely to be important for viability/fertility than others. In humans, transcription factor mutations appear enhanced in prenatal disease, and enzymes are overrepresented in diseases with onset in the first year after birth (Jimenez-Sanchez et al., 2001). We investigated four classes of protein identified by GO terms: transcription factors (n = 7), transmembrane proteins (n = 50), enzymes (n = 131), and chromatin-associated proteins (n = 24). Numbers of each were limited, but there was no significant enrichment for essential genes among any of the four groups (Tables S2 and S3).

In summary, we found that essential genes were less likely to have a paralog and more likely to be part of a protein complex, but no specific class of protein appeared more likely to be predictive of essentiality.

Annotating the Function of Novel Genes

There is a large bias in the literature toward analysis of known genes (Edwards et al., 2011), but are genes that have yet to be examined experimentally less likely to underlie disease? Genes in our set that had no associated publications (other than high-throughput genome-wide reports) were compared with genes where some aspect of their function had been described. The proportion of essential genes among the novel set was not significantly different from the known genes (Figure 7C). Furthermore, there was no significant difference in the number of hits observed per line between known and novel genes (Tables S2 and S3). As a second test, we asked if genes with orthologs involved in human disease (having an OMIM disease ID) were enriched in essential genes or the number of phenotypic hits compared with genes not (yet) ascribed to human disease, but there was no significant difference (Tables S2 and S3; Figure 7D). Finally, we compared genes that had been proposed for inclusion by the community (n = 87) with those with no specific request to ask if genes of interest to the community were more likely to be essential or to have detected phenotypes. There was no significant difference between the two groups (Table S3). Thus, known genes are no more likely to be involved in disease than novel genes, emphasizing that much new biology will be uncovered from the analysis of mutations in novel genes.

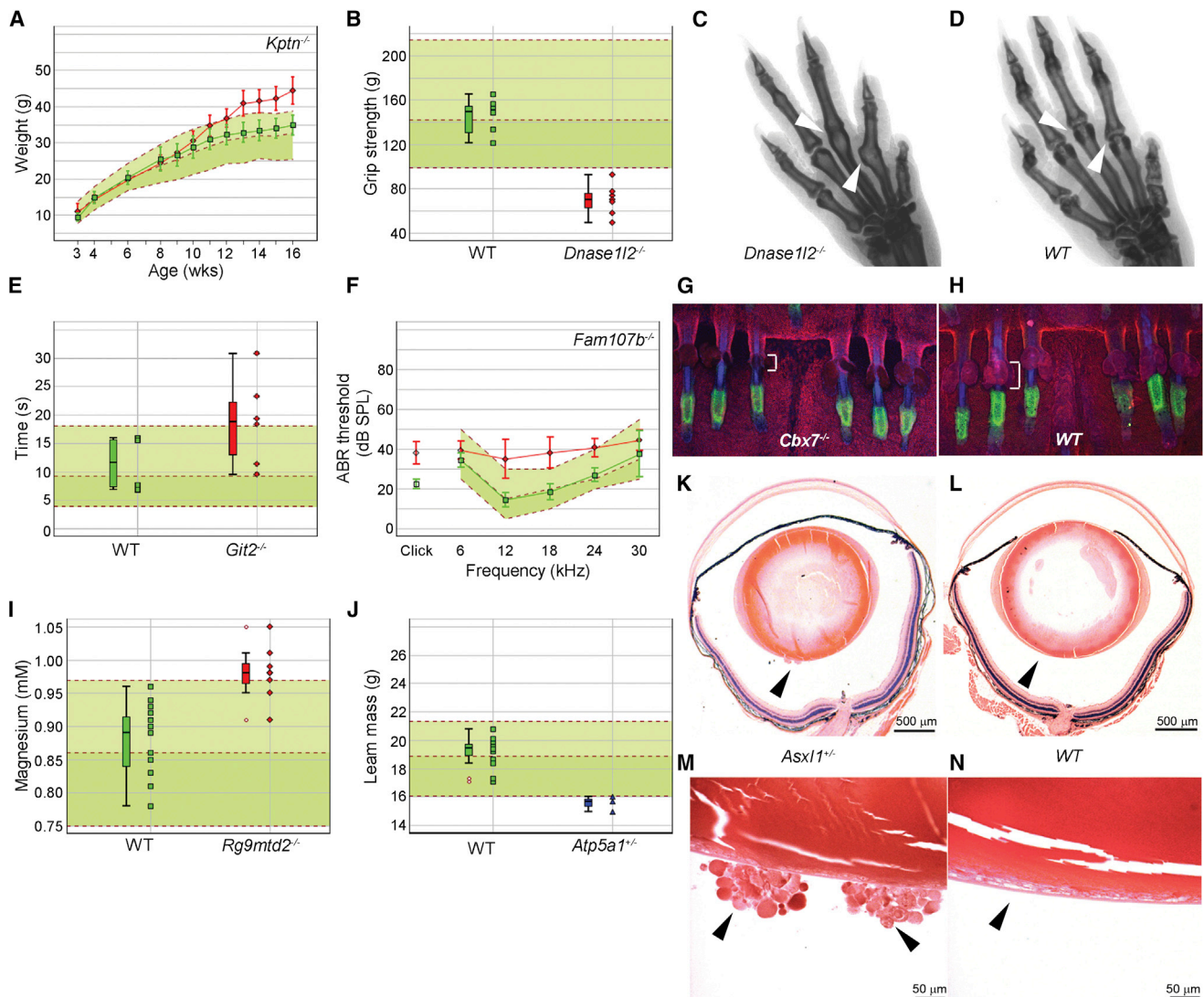


Figure 4. Examples of Novel Phenotypes from a Wide Range of Assays with Particular Focus on Novel Genes

(A) Elevated body weight gain of *Kptn*^{tm1a/tm1a} females (n = 7) fed a high-fat diet from 4 weeks of age. Mean \pm SD body weight is plotted against age for *Kptn*^{tm1a/tm1a} females (red line) and local WT controls run during the same weeks (n = 16; green line). The median and 95% reference range (2.5% and 97.5%; dotted lines) for all WT mice of the same genetic background and sex (n = 956 females) are displayed on the pale green background.

(B) Reduced grip strength in *Dnase1l2*^{tm1a/tm1a} males (n = 7) (red symbols) compared with controls (n = 8) (green symbols) and the reference range (n = 289). Each mouse is represented as a single symbol on the graph. Median, 25th and 75th percentile (box), and the lowest and highest data point still within 1.5 \times the interquartile range (IQR) (whiskers) are shown.

(C and D) Ankylosis of the metacarpophalangeal joints (arrowheads) shown by X-ray in *Dnase1l2*^{tm1a/tm1a} mice (C) (six of seven males; five of seven females) compared with WT controls (D) correlates with reduced grip strength (B).

(E) Increased latency to respond to heat stimulus in *Git2*^{Git(XG510)Byg1 Git(XG510)Byg} females (n = 6) (red symbols) compared with controls (n = 4) (green symbols) and the reference range (n = 115), with box and whisker plots on the left (see Figure 4B legend).

(F) Mild hearing impairment at the middle range of frequencies in *Fam107b*^{tm1a/tm1a} mutants (n = 8) (red line shows mean \pm SD) compared with controls (n = 10) and the reference range (n = 440).

(G) Smaller sebaceous glands (indicated by bracket) in *Cbx7*^{tm1a/tm1a} mutant tail skin hairs compared with WT (H).

(I) Increased plasma magnesium levels in *Rg9mtd2*^{tm1a/tm1a} males (n = 8) (red symbols) compared with local controls (n = 15) (green symbols) and the reference range (n = 241), with box and whisker plots on the left (see Figure 4B legend).

(J) Decreased lean mass in *Atp5a1*^{tm1a/+} females (n = 3) (blue symbols) compared with local controls (n = 15) (green symbols) and the reference range (n = 757), with box and whisker plots on the left (see Figure 4B legend).

(K and L) Histopathology showed opacities in the vitreous of eyes from *Asx1*^{tm1a/+} mice (K) (arrowheads; scale bar, 500 μ m) compared with empty vitreous in WT (L).

(M and N) Higher magnification revealed round opacities extending from the lens into the vitreous (arrowheads; scale bar, 50 μ m) in *Asx1*^{tm1a/+} mice (M) compared with a normal lens contained within the lens capsule in WT mice (N).

See also Table S5.

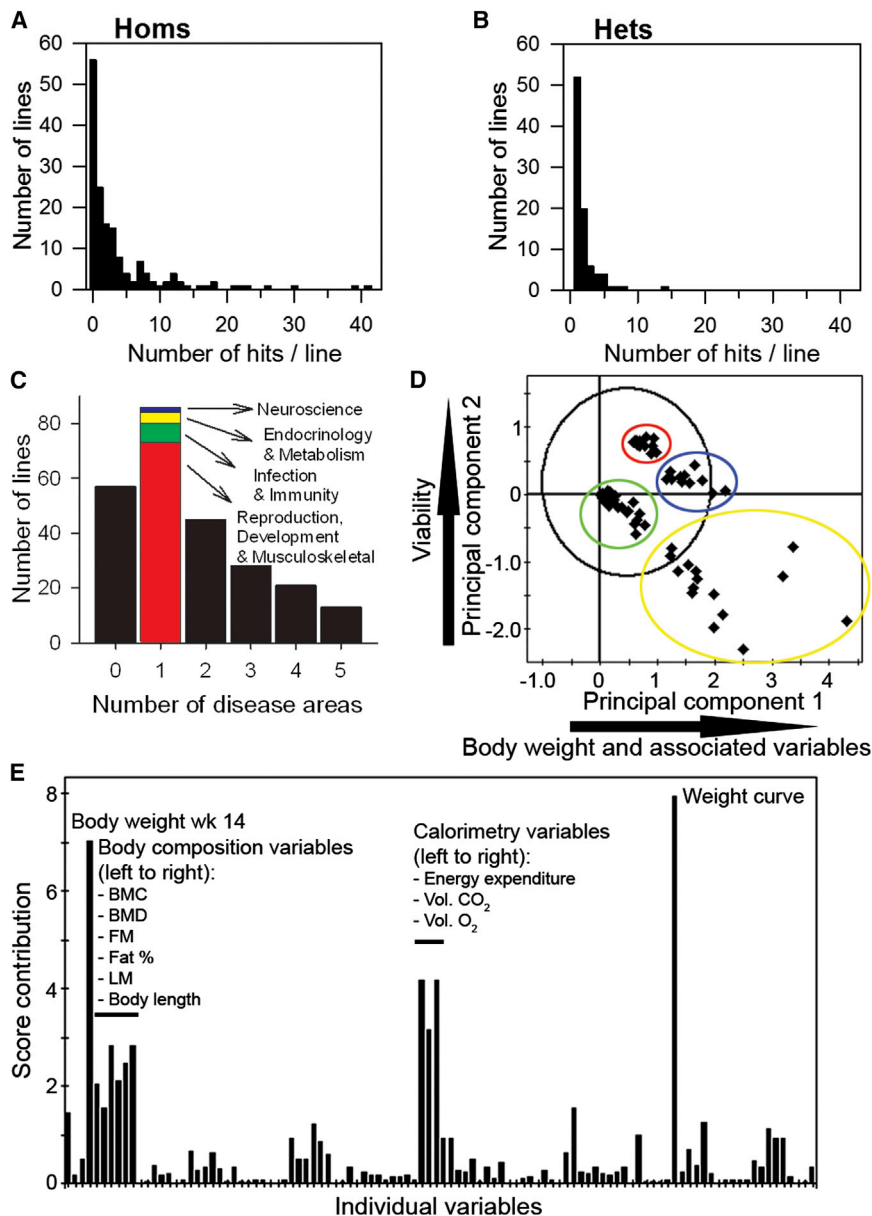


Figure 5. Characteristics of Phenotypic Hits Detected

(A) Distribution of the number of phenotypic hits in each line screened as homozygotes showing the peak at no hits but a long tail of lines with multiple hits up to 41.

(B) Distribution of hits in lines screened as heterozygotes; all lines had at least one hit (for viability) with a spread up to 14 hits.

(C) Distribution of lines with hits in different disease areas showing a peak of lines with just one area affected (colors indicate which areas) but some lines with multiple disease areas involved, indicating a high degree of pleiotropy.

(D) Principal component analysis score scatterplot showing the deviation of each gene from the first two principal components to visualize the clustering in genes within the multidimensional space. The black ovoid represents the Hotelling's T^2 95% confidence limits. Colored ovoids mark four different clusters of mutant lines. The two main principal components (or latent variables) in the model are significant in explaining 19.2% and 11.7% of the variation, respectively, and are predictive.

(E) Principal component analysis contribution plot indicating the contribution of the variables to the separation between the red and green clusters compared to the blue and yellow clusters in (D). Major phenotypic contributions are labeled.

Key to variables is presented in Table S7.

standard allele design with the option of generating conditional mutations, and all are available from public repositories.

The phenotyping described here was not intended to provide an exhaustive characterization of the phenotype of the mutant lines but, rather, to place mutant alleles into broad categories by using screens, generating a pool of genetic resources from which individual mutants can be selected based on their phenotype for secondary follow-up studies. Several of the mutants have been

analyzed further following an initial phenotypic observation in the screen, and these add to the depth of our knowledge of biological mechanisms of disease (e.g., Nijnik et al., 2012; Crossan et al., 2011). As the assembled data expands, it will become possible to discern patterns between phenotypes and come to more holistic conclusions about categories of genes. Genes linked by common phenotypes can be grouped together to test for regulatory or other functional interactions and ultimately placed into pathways that in turn will implicate other genes in the disease process. For example, of the four genes associated with abnormal fasting glucose levels in our data set, *Slc16a2* can be linked to *Ldha* via regulation of L-triiodothyronine (Friesema et al., 2006; Miller et al., 2001), but the other two genes, *Nsun2* and *Cyb561*, have no reported regulatory links apart from in vitro protein-protein interactions, so these represent

DISCUSSION

Genetic studies in mice via targeted mutagenesis of ES cells have been successful at illuminating selected aspects of the function of more than 7,000 mammalian genes. However, until recently, these studies have been conducted by individual laboratories and largely directed at previously studied genes. The focused collection of phenotypic information from these mutants has been very information rich, but many aspects remain undetected because they are outside the area of interest of the laboratory generating the mutant. Individual endeavors have led to wide variation in allele design and genetic backgrounds used, and all too often, the mutant is not available to other groups for further analysis. In contrast, the mutant mice described here have the advantage of a common genetic background and a

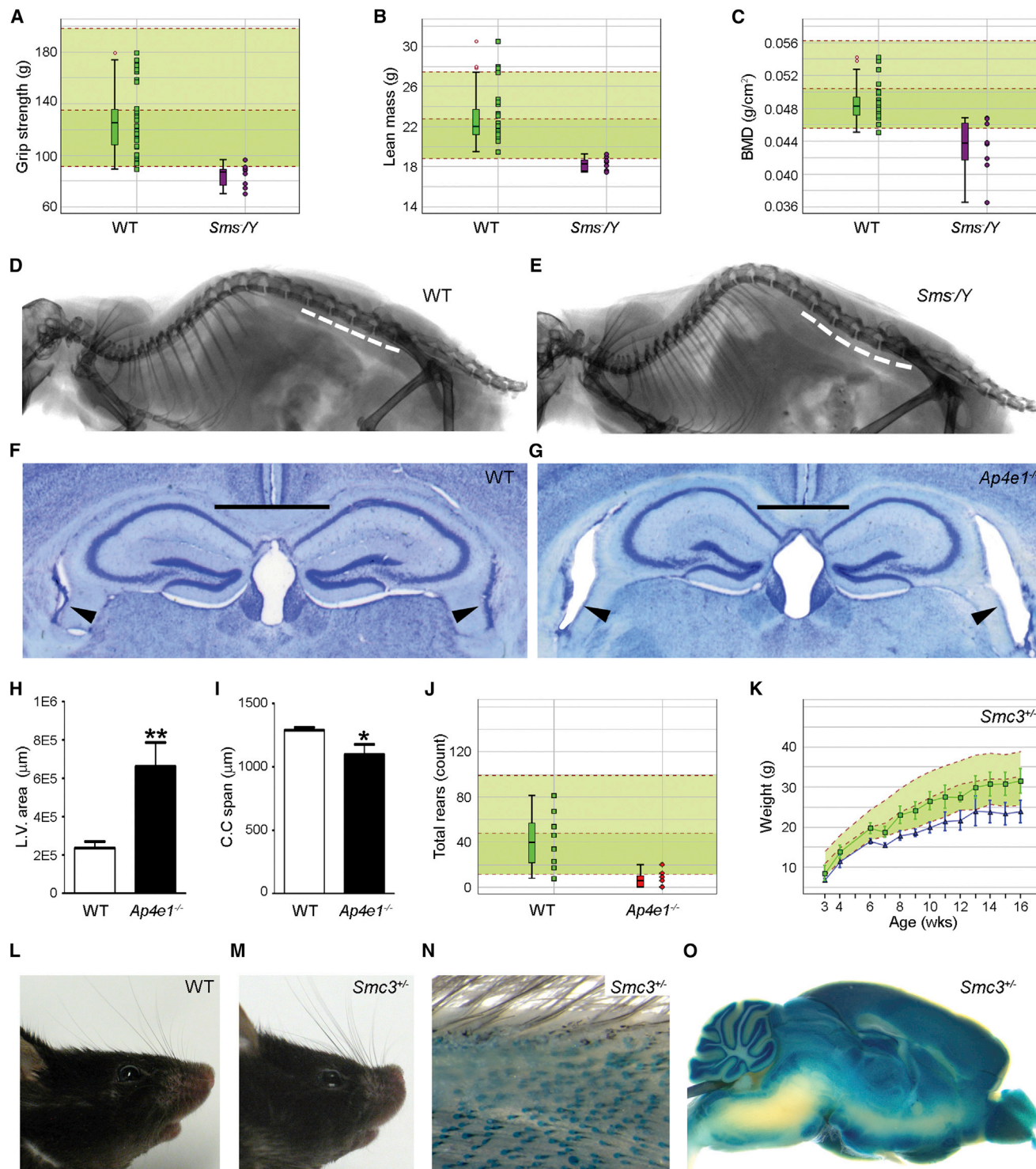


Figure 6. Correlated Disease Characteristics in Knockouts of Three Known Human Disease Genes

(A–E) Male hemizygotes for the *Sms* mutation showed similar features to X-linked Snyder-Robinson syndrome.

(A) Reduced grip strength in *Sms/Y* mice ($n = 8$) (purple symbols) compared with WT controls ($n = 30$) (green symbols) and the reference range ($n = 793$). Each mouse is represented as a single symbol on the graph, with box and whisker plots on the left (see Figure 4B legend).

(B and C) Decreased lean mass (B) and bone mineral density (C) in *Sms/Y* mice ($n = 8$) (purple symbols) compared with controls ($n = 27$) (green symbols) and the reference range ($n = 753$), with box and whisker plots on the left (see Figure 4B legend).

(D and E) Lumbar lordosis shown by X-ray (seven of eight males) in *Sms/Y* (E) compared with WT (D).

(legend continued on next page)

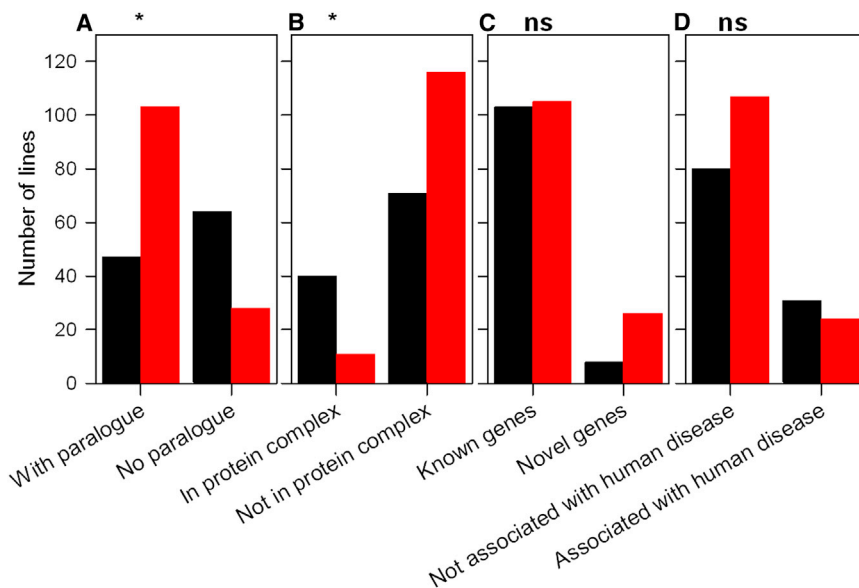


Figure 7. Features Associated with Essential Genes

Essential genes (black bars) are compared with genes that are not essential for viability (red bars). The asterisk (*) indicates significant difference. ns, no significant difference in proportion of essential genes between the two categories. Statistics are presented in Table S3.

(A) Genes with no paralog show a significantly larger proportion of essential lines than genes with at least one paralog.

(B) Genes predicted to contribute to protein complexes showed a significantly larger proportion of essential lines than genes not predicted to contribute to a complex.

(C) Novel genes showed no significant difference in proportion of essential genes or number of hits than known genes.

(D) Genes known to underlie human disease were no more likely to be essential than genes not yet associated with human disease.

candidates to investigate further. Already some broad conclusions can be drawn from the data set, such as the value of analyzing novel genes, the increased incidence of essentiality in genes with no paralog, and the increased number of genes required for male compared to female fertility. Many completely unexpected associations between genes and phenotypes have been discovered, illustrating the value of a broad-based screen.

Another aspect of our study was the examination of heterozygous mutants, a genotype that often is not studied by individual laboratories. Although this was restricted to mutants that displayed lethality or subviability of homozygotes, it revealed a number of genes with haploinsufficiency, a feature commonly associated with mutations in the human genome but rarely described in mouse knockouts.

The tests used in screening varied considerably in their complexity, cost, and suitability in a high-throughput scenario. The performance of these tests across 250 alleles provided insight into those that should be included or excluded in the efforts to examine 5,000 alleles through the activities of the IMPC. Key considerations are variance in the control group, specificity, sensitivity, effect size, and redundancy.

The major contribution of null alleles will be an improved understanding of biological processes and molecular mechanisms of disease. The null allele will give insight into the temporal and spatial requirements for the gene and will contribute to the establishment of gene networks involved in mammalian disease processes. Furthermore, our data set demonstrates that many features of human Mendelian diseases can be found in the corresponding mouse mutant. The mouse alleles studied here are expected to be null alleles or strong hypomorphs, which may not always reflect the consequence of the human mutation. However, null alleles should reveal haploinsufficiency and recessive effects due to deleterious mutations such as frameshift and nonsense mutations. Null alleles in the mouse are likely to make the largest impact upon understanding human diseases caused by rare variants of large effect size. Complex multifactorial diseases, which may depend on human-specific variants with small effect size or more specific molecular effects such as gain-of-function mutations, will require more customized approaches such as knockin of specific human mutations. Alternative approaches using the mouse for discovering loci underlying complex disease include the Hybrid Mouse Diversity panel and the Collaborative Cross (reviewed by Flint and Eskin, 2012).

(F–J) *Ap4e1^{tm1a/tm1a}* mice displayed similarities to spastic quadriplegic cerebral palsy 4.

(F–I) Increased lateral ventricle area (arrowheads in F and G) and decreased corpus callosum span (solid lines in F and G) in *Ap4e1^{tm1a/tm1a}* mice (G) compared with WT mice (F) with measurements plotted (mean \pm SD) in (H) and (I), respectively (* $p < 0.05$, ** $p < 0.01$; $n = 3$ mutant males and 34 WT males). Error bars in (H) and (I) are SD.

(J) Decreased rearing in *Ap4e1^{tm1a/tm1a}* females ($n = 7$) (red symbols) compared with WT controls ($n = 8$) (green symbols) and the reference range ($n = 180$), with box and whisker plots on the left (see Figure 4B legend).

(K–O) Surviving *Smc3^{tm1a/+}* mice showed similar features to Cornelia de Lange syndrome 3.

(K) Decreased body weight in *Smc3^{tm1a/+}* females ($n = 7$) fed on high-fat diet. Mean \pm SD body weight is plotted against age for *Smc3^{tm1a/+}* females (blue line), WT mice ($n = 24$; green line), and the reference range ($n = 948$).

(L and M) Distinct craniofacial abnormalities in *Smc3^{tm1a/+}* mice including upturned snout (M) (three of seven males, one of seven females), which was not observed in WT mice (L) ($n = 850$ male and 859 female).

(N and O) The *lacZ* reporter gene revealed a distinct *Smc3* expression pattern including (N) hair follicles and (O) key brain substructures, noteworthy because of the hirsutism and neurodevelopmental delay aspects of Cornelia de Lange syndrome 3.

See also Table S6.

These allow interrogation of many different loci simultaneously and study of epistatic interactions and can lead to identification of single gene variants causing disease (e.g., Orozco et al., 2012; Andreux et al., 2012), when variants affecting the trait of interest are present in the founders.ENU mutagenesis is another powerful technique that can be used to produce allelic series of mutations with differing effects upon function of single genes (e.g., Andrews et al., 2012). However, the null alleles that we describe here are a complement to these alternative approaches and will be invaluable for defining mechanisms of gene function on a standard genetic background.

The study described in this report builds on the large KOMP/EUCOMM resource of targeted mutations in mouse ES cells (Skarnes et al., 2011) and illustrates the breadth of phenotypic information that can be garnered from an organized effort. The Clinical Phenotyping Pipeline optimized here has been adopted by several other programs within the IMPC; multiple groups are now working together to extend what is described in this report for 250 genes to 5,000 genes over the next 4 years with the vision that this will eventually cover all protein-coding genes. The primary phenotypes and genetic resources emerging from these programs will make a significant contribution to our understanding of mammalian gene function.

EXPERIMENTAL PROCEDURES

Animals

Mice carrying knockout first conditional-ready alleles (Figures S1A and S1B) were generated from the KOMP/EUCOMM targeted ES cell resource using standard techniques. Eight in-house lines were included as known mutant controls. Details of the 250 lines can be found in Table S2. All lines are available from <http://www.knockoutmouse.org/>; or mouseinterest@sanger.ac.uk. Mice were maintained in a specific pathogen-free unit under a 12 hr light, 12 hr dark cycle with ad libitum access to water and food. The care and use of mice were in accordance with the UK Home Office regulations, UK Animals (Scientific Procedures) Act of 1986.

Genotyping and Allele Quality Control

Short-range, long-range and quantitative PCR strategies (<http://www.knockoutmouse.org/kb/25/>) were used to evaluate the quality of each allele (Figure S1C). A subset of these assays was used to genotype offspring. The degree of knockdown in homozygotes was assessed by qRT-PCR of adult liver in a subset known to show expression in liver. Details are given in Extended Experimental Procedures.

Phenotyping Pipeline and Tests

The typical workflow from chimera to primary phenotyping pipelines and an outline of the clinical phenotyping pipeline are presented in Figure 1. Details of batch size are given in Figure S2A. These pipelines include established tests used to characterize systematically every line of mice as described in Table S4.

Histochemical Analysis of the *lacZ* Reporter

Adult whole-mount *lacZ* reporter gene expression was carried out essentially as described by Valenzuela et al. (2003).

Statistical and Bioinformatic Analysis

For continuous data, including time course, a reference range approach was used to identify phenotypic variants as detailed in Figures S3A and S3B. Fisher's exact test was used to assess categorical data (Figure S3C). These automated calls were complemented by a manual assessment made by biological experts. An example of the establishment of the reference range is given in Figure S2B.

Downstream data analysis was performed using SPSS (version 17.0.2), R, and SIMCA-P (V-12.0, Umetrics). The data structure and biological question determined the statistical test used; details are in Table S3. Principal components analysis was performed in SIMCA-P (<http://www.umetrics.com>).

Further details of analyses and gene annotations are given in Extended Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, three figures, seven tables, and a complete list of contributors from the Sanger Institute Mouse Genetics Project and can be found with this article online at <http://dx.doi.org/10.1016/j.cell.2013.06.022>.

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