Highly variable penetrance of abnormal phenotypes in embryonic lethal knockout mice [version 2; peer review: 3 approved]

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Abstract

Background: Identifying genes that are essential for mouse embryonic development and survival through term is a powerful and unbiased way to discover possible genetic determinants of human developmental disorders. Characterising the changes in mouse embryos that result from ablation of lethal genes is a necessary first step towards uncovering their role in normal embryonic development and establishing any correlates amongst human congenital abnormalities.

Methods: Here we present results gathered to date in the Deciphering the Mechanisms of Developmental Disorders (DMDD) programme, cataloguing the morphological defects identified from comprehensive imaging of 220 homozygous mutant and 114 wild type embryos from 42 lethal and subviable lines, analysed at E14.5.

Results: Virtually all mutant embryos show multiple abnormal phenotypes and amongst the 42 lines these affect most organ systems. Within each mutant line, the phenotypes of individual embryos form distinct but overlapping sets. Subcutaneous edema, malformations of the heart or great vessels, abnormalities in forebrain morphology and the musculature of the eyes are all prevalent phenotypes, as is loss or abnormal size of the hypoglossal nerve.

Conclusions: Overall, the most striking finding is that no matter how
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profound the malformation, each phenotype shows highly variable penetrance within a mutant line. These findings have challenging implications for efforts to identify human disease correlates.

Keywords
mouse, embryo, phenotype, morphology, high-resolution episcopic microscopy, development, penetrance
Introduction

Animal models have long been used as experimental surrogates for investigating the role of individual genes in human development and disease. The remarkable degree of conservation in gene sequence and role that we now know exists across species confirms the validity of this approach and genetic manipulation in the mouse provides a commonly used way to explore gene function. The most ambitious example of this is the attempt coordinated by the International Mouse Phenotyping Consortium (IMPC) to generate a catalogue of gene function, using a systematic approach to phenotyping of individual gene knockouts (KO) that cover the entire mouse genome. In generating KO lines from about one quarter of the total mouse genome so far, these studies have revealed that around one third of all mammalian genes are essential for life\textsuperscript{1,2}, their removal resulting in embryonic or perinatal lethality. The study of such mutant lines provides a unique opportunity to gain a comprehensive overview of the genetic components regulating normal embryo development and, by inference, the identity of genes whose mutation may cause congenital abnormalities or developmental disease.

Deciphering the Mechanisms of Developmental Disorders (DMDD) is a five year, UK-based programme funded by the Wellcome Trust with the goal of studying 240 embryonic lethal KO lines\textsuperscript{1}. By applying systematic phenotyping methods for homozygous mutant embryos with parallel efforts to identify placental abnormalities and changes in early embryo transcriptome profiles, DMDD offers a foundation for identifying novel genes important for developmental or clinical studies. Here we summarise results to date from detailed examination of homozygous mutant embryos at E14.5 for structural abnormalities.

Materials and methods

Embryos

All embryos were produced by the Wellcome Trust Sanger Institute (https://www.sanger.ac.uk/mouseportal/) as part of the DMDD project\textsuperscript{1}. Gene knockout lines produced as part of a systematic programme coordinated by the International Mouse Phenotyping Consortium (http://www.mousephenotype.org) were designated lethal if no homozygous mutants were present amongst a minimum of 28 pups at P14 and sub-viable if their proportion fell below 13% of total offspring\textsuperscript{2}. All embryos are obtained from heterozygous intercrosses independently from the P14 viability call. Embryos were harvested from one or more litters at E14.5, fixed in Bouin’s fixative for 24 hours and stored at 4°C in phosphate buffered saline.

Generation of digital volume data

Embryos were initially scored for gross abnormalities under a dissection microscope before preparation for 3D imaging. Briefly, embryos were dehydrated in methanol (10% steps until 90%, followed by 95% and 100%; at least 2 hours each) and embedded in methacrylate resin (JB-4, Polysciences) containing eosin B and acridine orange, as previously described\textsuperscript{3,4}. Within each resin block, the embryo was oriented to ensure transverse sectioning along its longitudinal axis. Resin blocks were allowed to polymerise overnight at room temperature, baked at 90°C for 24–48 hours and then subjected to digital volume data generation using high-resolution episcopic microscopy (HREM)\textsuperscript{5}. HREM data was downsized as appropriate to provide an isotropic voxel size of between 2.5–3 μm, depending on original section thickness.

Data processing and annotation

12 bit raw greyscale image data was adjusted to optimise tissue visualisation using Photoshop 6 (Adobe). Data visualisation and analysis was performed using software packages Amira 5 (ThermoFisher Scientific) and Osirix, versions 6–8 (Pixmeo). Phenotypes were identified by establishing the precise developmental sub-stage of each embryo and comparing it with stage-matched controls\textsuperscript{5}. Phenotyping was performed according to a standardised and sequential procedure using actual and virtual 2D section stacks, essentially as recently described\textsuperscript{5}. Data from each embryo was independently reviewed by a second anatomist, and any discrepancies resolved by joint agreement. Each phenotype call was assigned to a 3D point within the embryo image data stack. Abnormalities were classified with the Mammalian Phenotype (MP) ontology\textsuperscript{6}, using the most specific MP term that described each defect. 3D volume rendered models were employed for developmental staging from external morphology\textsuperscript{6}.

Data analysis

In order to facilitate summarising of detailed phenotype annotation data, two subsets of the MP terms closer to the root of the ontology were chosen to provide structured “high” and “intermediate” level overviews of DMDD phenotype data. These MP ontology slims are shown in Table 5 and Table 6 (Supplementary Table 2 and Supplementary Table 3 for download). The MP terms assigned during annotation of the embryos were summarised into the categories defined by the DMDD slims using the Map2Slim algorithm (https://metacpan.org/pod/distribution/go/perl/scripts/map2slim). All the terms of the DMDD slims that map to terms used to annotate mutant and wild type embryo phenotypes are listed in Supplementary Table 1A and Supplementary Table 1B, respectively.

MP annotation terms used to describe the phenotypes of each embryo of a line were normalised to remove duplicate terms, and the terms for each embryo were mapped onto the ontology slims. For each line, a set of the unique slim terms observed for the line was generated and lists were produced of all the embryos from the line falling into each of these high or intermediate level categories. This enabled calculation of a penetrance score for each of the broad slim terms, calculated as a ratio of the number of embryos listed for the slim category to the number of homozygous mutant embryos in the line falling into each of these high or intermediate level categories.

To obtain a global view of the phenotypes detected, the frequency of lines showing each of the broad category slim terms were counted across all the lines analysed. In addition, the incidence of embryos scored for every phenotype category described by the slim terms,
and the total number of embryos analysed in lines exhibiting each individual phenotype category was counted.

The total number of lines for each slim term that had a penetrance score between 0–0.24, 0.25–0.49, 0.50–0.74 and 0.75–1.00 was recorded. We calculated the cumulative penetrance score for each slim term as the overall sum of the penetrance scores of every line showing this broad category phenotype. In addition, for each of the penetrance intervals listed above, the sum of the penetrance scores was calculated for the lines falling into these categories.

All plots showing analysis of the data were produced using the R software package, version 3.2.1 (2015-06-18) (The R Foundation for Statistical Computing).

Use of animals
The care and use of all mice in this study were in accordance with UK Home Office regulations, UK Animals (Scientific Procedures) Act of 1986 (PPL 80/2485) and were approved by the Welcombe Trust Sanger Institute’s Animal Welfare and Ethical Review Body.

Results
Size of the study
The data for this study comprises 220 homozygous mutant and 114 wild type E14.5 embryos analysed by the DMDD programme. All data is presented in Supplementary Table 4 and Supplementary Table 5 and is and also available on the DMDD web site (https://dmdd.org.uk). Embryos were obtained from 42 novel gene knockout lines, 31 classified as lethal and 11 as sub-viable (Table 1; see also Materials and methods). This corresponds to an average of approximately 5 homozygous mutant embryos for each mutant line, although in practice numbers ranged widely from 1 to 11 as a result of variable breeding efficiency and cost limitations inherent in a large scale screening programme (Supplementary Figure 1). In total, 1,128,247 transverse section images obtained from the 334 embryos formed the basis for examining embryo structure and with the addition of digital resection of datasets in coronal and sagittal planes, scoring of phenotypes was based on examination of 2,536,659 images.

Incidence of structural abnormalities in homozygous mutant embryos.
Almost all mutant embryos studied (209/220) showed structural abnormalities that could be identified by a phenotyping procedure previously refined from pilot studies6. The remaining 11 apparently normal embryos were obtained from 9 different lines, each of which yielded several other homozygous mutants bearing detectable morphological abnormalities. We have previously reported that the resolution afforded by 3D datasets obtained by HREM imaging allowed the detection of phenotypic abnormalities spanning in size range from individual nerves and blood vessels to gross organ and tissue malformations6. In the present study, a total of 398 different MP terms were employed to record a total of 2,939 detected mutant embryo phenotypes (Table 2A and Supplementary Table 1A and Supplementary Table 4). Multiple abnormalities were scored in virtually all homozygous mutant embryos. Most showed up to 10, but in some embryos as many as 50 phenotypes were recorded (Figure 1A). Whilst a few phenotypes (for example those affecting different parts of vertebrae or different regions of the vertebral column) were often scored repeatedly within affected embryos, their incidence was insufficient to have a significant impact on the overall distribution of phenotype numbers scored per embryo across the whole study. When analysed by individual mutant line, the incidence of detectable abnormalities is more broadly distributed, with more than half of the 42 lines showing between 10 and 49 different phenotypes (Figure 1B).

Incidence of structural abnormalities in wild type embryos
To establish the possible impact of “background” abnormalities present within embryos irrespective of mutation, we also analysed a total of 114 wild type embryos, obtained from 41 of the 42 mutant lines (Table 1). Previous large-scale studies of wild type E14.5 embryos from the same genetic background have enabled us to distinguish normal variation in structure from definite abnormalities, using careful stage-specific comparisons combined with statistical and morphometric analysis6. This formed the basis for identifying phenotypes in the wild type embryos (Table 2B and Supplementary Table 1B and Supplementary Table 5).

In total, 56 phenotype calls were made, affecting 32 of the wild type embryos and 28 of the 41 lines. 21 of the 56 phenotype calls (38%) are accounted for by only 6 embryos, (indicating the skewing effect of a small number of abnormal embryos). Most affected embryos showing only a single phenotype. This is in marked contrast to the finding of many different phenotypes in individual mutant embryos.

The phenotypes of wild types vary in character, ranging from apparently minor differences (e.g. in blood vessel morphology) to a few major abnormalities (e.g. absent kidney). Each one is rare amongst the population of wild type embryos analysed and affects only a single wild type embryo within the line. Only 10 phenotypes (15 phenotype calls) overlap between mutant embryos and their wild type siblings and these affect only 10 of the 41 lines for which wild type embryos have been assessed (Table 3).

Prevalence of individual abnormalities in mutant embryos
Supplementary Table 1A presents the frequency of individual abnormalities that were identified amongst the mutant embryos. Since some phenotypes (such as vertebral abnormalities) are often present multiply in affected embryos, the data is normalised for occurrence by embryo. Interestingly, the most common phenotype detected in this study was subcutaneous edema. This was evident from macroscopic observation of embryos at harvest and confirmed by subsequent HREM imaging (Figure 3, panels A–C). In total, subcutaneous edema and edema in other body regions (scored with four distinct MP terms) affected one third (72/220) of the embryos and was observed in a little over half (24/42) of the mutant lines. Other prevalent phenotypes included defects affecting the vertebral arches, the ventricular septum of the heart, forebrain morphology and musculature of the developing eyes (Table 2A and Figure 3). Of particular note is the frequency with which mutant embryos showed abnormalities affecting the architecture or presence of the hypoglossal nerve (Figure 4, panels A and B). Complete absence of the nerve occurred in 37 embryos, obtained from 12 different mutant lines, with some embryos from a similar number of lines
### Table 1. List of lethal and subviable lines studied.

The gene symbol, Mouse Genome Informatics (MGI) ID for the gene, and allele symbol is listed for each line studied along with the number of homozygous mutant embryos analysed, genetic background and the viability status.

| Gene    | MGI ID | Allele                  | Genetic Background | E14.5 homozygous mutant embryos analysed | E14.5 homozygous viability |
|---------|--------|-------------------------|--------------------|------------------------------------------|---------------------------|
| P14     |        |                         |                    |                                          |                           |
| E14.5   |        |                         |                    |                                          |                           |
| 170007K01Rik | MGI:1920733 | 170007K01Rik<tm2a(KOMP)Wtsi> | C57BL/6J;C57BL/6NTac | 8                          | Lethal                    |
| 49334920Rik | MGI:1914027 | 49334920Rik<tm1a(EUCOMM)Wtsi> | C57BL/6J;C57BL/6NTac | 6                          | Lethal                    |
| 1098490 | MGI:1932407 | 1098490<tm1a(EUCOMM)Wtsi> | C57BL/6J;C57BL/6NTac | 7                          | Lethal                    |
| 1700067K01Rik | MGI:1920703 | 1700067K01Rik<tm2a(KOMP)Wtsi> | C57BL/6J;C57BL/6NTac | 2                          | Lethal                    |
| 49334920Rik | MGI:1914027 | 49334920Rik<tm1a(EUCOMM)Wtsi> | C57BL/6J;C57BL/6NTac | 6                          | Lethal                    |
| 1098490 | MGI:1932407 | 1098490<tm1a(EUCOMM)Wtsi> | C57BL/6J;C57BL/6NTac | 7                          | Lethal                    |
| 1700067K01Rik | MGI:1920703 | 1700067K01Rik<tm2a(KOMP)Wtsi> | C57BL/6J;C57BL/6NTac | 2                          | Lethal                    |
| Gene  | MGI ID | Allele                                                                 | Genotype | Viability     | E14.5 wildtype embryos analysed |
|-------|--------|------------------------------------------------------------------------|----------|---------------|-------------------------------|
| Nsun2 | MGI:107252 | Nsun2<tm1a(EUCOMM)Wtsi>                                              | Subviable| 6             |                               |
| Nro   | MGI:109331 | Nro<tm1b(EUCOMM)Wtsi>                                                 | Lethal   | 3             |                               |
| Ctubd7b | MGI:1923901 | Polb<tm1a(EUCOMM)Wtsi>                                               | Lethal   | 1             |                               |
| Pdzk1 | MGI:1928759 | Pdzk1<tm1b(EUCOMM)Wtsi>                                              | Subviable| 9             |                               |
| Polb  | MGI:1928801 | Polb<tm2b(EUCOMM)Wtsi>                                               | Lethal   | 1             |                               |
| P14    |         |                                                                       |          |               |                               |
| Nsun2 | MGI:107252 | Nsun2<tm1a(EUCOMM)Wtsi>                                              | Subviable| 6             |                               |
| Nro   | MGI:109331 | Nro<tm1b(EUCOMM)Wtsi>                                                 | Lethal   | 3             |                               |
| Ctubd7b | MGI:1923901 | Polb<tm1a(EUCOMM)Wtsi>                                               | Lethal   | 1             |                               |
| Pdzk1 | MGI:1928759 | Pdzk1<tm1b(EUCOMM)Wtsi>                                              | Subviable| 9             |                               |
| Polb  | MGI:1928801 | Polb<tm2b(EUCOMM)Wtsi>                                               | Lethal   | 1             |                               |
Table 2A. Frequency of phenotypes identified in homozygous mutant embryos. The Mammalian Phenotype Ontology terms describing phenotypes observed in each embryo were normalised to remove duplicates and the list then ranked in descending order by frequency of embryos exhibiting each phenotype.

| MP ID   | MP term                                      | Frequency |
|---------|----------------------------------------------|-----------|
| MP:0013848 | subcutaneous edema                         | 64        |
| MP:0004613 | fusion of vertebral arches                  | 61        |
| MP:0010418 | perimembraneous ventricular septal defect   | 49        |
| MP:0000783 | abnormal forebrain morphology              | 47        |
| MP:0003686 | abnormal eye muscle morphology             | 45        |
| MP:0010115 | small superior cervical ganglion            | 45        |
| MP:0010420 | muscular ventricular septal defect          | 41        |
| MP:0013835 | absent hypoglossal nerve                     | 37        |
| MP:0000782 | abnormal Mullerian duct morphology         | 33        |
| MP:0014021 | heterochrony                                | 33        |
| MP:0004269 | abnormal optic cup morphology               | 32        |
| MP:0014001 | abnormal vertebral artery topology          | 32        |
| MP:0013836 | abnormal hypoglossal nerve topology         | 30        |
| MP:0013876 | absent ductus venosus valve                 | 29        |
| MP:0000284 | double outlet right ventricle               | 29        |
| MP:0004666 | absent stapedial artery                     | 28        |
| MP:0013971 | blood in lymph vessels                      | 27        |
| MP:0000703 | abnormal thymus morphology                  | 26        |
| MP:0014000 | anastomosis between internal carotid artery and basilary artery | 25        |
| MP:0000602 | enlarged liver sinusoidal spaces            | 25        |
| MP:0013969 | reduced sympathetic cervical ganglion size  | 25        |
| MP:0008923 | thoracochisis                               | 25        |
| MP:0004163 | abnormal adenohypophysis morphology         | 24        |
| MP:0002237 | abnormal nasal cavity morphology            | 20        |
| MP:0013986 | abnormal vitelline vein topology            | 20        |
| MP:0013967 | abnormal infrahyoid muscle connection       | 18        |
| MP:0004463 | basi spheno id bone foramen                | 18        |
| MP:0008128 | abnormal brain internal capsule morphology  | 16        |
| MP:000282  | abnormal interatrial septum morphology     | 16        |
| MP:0004268 | abnormal optic stalk morphology             | 16        |
| MP:0013936 | abnormal thymus topology                    | 16        |
| MP:0014017 | abnormal Wolffian duct connection           | 15        |
| MP:0013877 | abnormal ductus venosus valve morphology    | 15        |
| MP:0002239 | abnormal nasal septum morphology           | 15        |
| MP:0000497 | abnormal small intestine placement          | 15        |
| MP:0001111 | cleft palate                                | 15        |
| MP:0013859 | abnormal vitelline vein connection          | 14        |
| MP:0013826 | absent hypoglossal canal                    | 14        |
| MP:0013840 | absent segment of posterior cerebral artery | 14        |
| MP:0013875 | trigeminal neura                            | 14        |
| MP:0010496 | abnormal pectinate muscle morphology       | 13        |
| MP:0013834 | thin hypoglossal nerve                      | 13        |
| MP:0003827 | abnormal Wolffian duct morphology          | 12        |
| MP:0013842 | ductus venosus stenosis                    | 12        |
| MP:0010912 | herniated liver                            | 12        |
| MP:0013968 | multiple persisting craniopharyngeal ducts | 12        |
| MP:0011361 | pelvic kidney                               | 12        |
| MP:0010572 | persistent right dorsal aorta              | 12        |
| MP:0002633 | persistent truncus arteriosis               | 12        |
| MP:0013931 | abnormal olfactory bulb position           | 11        |
| MP:0011683 | dual inferior vena cava                    | 11        |
| MP:0000914 | exencephaly                                | 11        |
| MP:0002169 | no abnormal phenotype detected              | 11        |
| MP:0000154 | rib fusion                                  | 11        |
| MP:0001611 | scoliosis                                   | 11        |
| MP:0004110 | transposition of great arteries            | 11        |
| MP:0012303 | umbilical vein stenosis                     | 11        |
| MP:0008922 | abnormal cervical rib                       | 10        |
| MP:0009917 | abnormal hyoid bone morphology             | 10        |
| MP:0009770 | abnormal optic chiasm morphology           | 10        |
| MP:0013844 | abnormal perichondrial ossification         | 10        |
| MP:0003345 | decreased rib number                       | 10        |
| MP:0011493 | double ureter                               | 10        |
| MP:0000445 | short snout                                 | 10        |
| MP:0002951 | small thyroid gland                        | 10        |
| MP:0013878 | abnormal ductus venosus valve topology      | 9         |
| MP:0000841 | abnormal hindbrain morphology              | 9         |
| MP:0010490 | abnormal inferior vena cava valve morphology | 9        |
| MP:0010853 | abnormal lung position or orientation      | 9         |
| MP:0001411 | abnormal vertebral body morphology         | 9         |
| MP:0002243 | abnormal vomeronasal organ morphology      | 9         |
| MP ID     | MP term                                                                 | Frequency |
|-----------|--------------------------------------------------------------------------|-----------|
| MP:0013970 | absent connection between subcutaneous lymph vessels and lymph sac       | 9         |
| MP:0011667 | double outlet right ventricle with atrioventricular septal defect        | 9         |
| MP:0014019 | embryo cyst                                                              | 9         |
| MP:0013977 | symmetric azygos veins                                                   | 9         |
| MP:0002092 | abnormal eye morphology                                                  | 8         |
| MP:0014023 | abnormal intestine placement                                             | 8         |
| MP:0001303 | abnormal lens morphology                                                 | 8         |
| MP:000632  | abnormal pineal gland morphology                                         | 8         |
| MP:0010602 | abnormal pulmonary valve cusp morphology                                 | 8         |
| MP:0013985 | abnormal umbilical vein topology                                          | 8         |
| MP:0013965 | abnormally deep median sulcus of tongue                                  | 8         |
| MP:0010484 | bicuspid aortic valve                                                    | 8         |
| MP:0004646 | decreased cervical vertebrae number                                      | 8         |
| MP:0010436 | abnormal brachial plexus formation                                       | 7         |
| MP:0013932 | abnormal coronary sinus morphology                                       | 7         |
| MP:000819  | abnormal olfactory bulb morphology                                       | 7         |
| MP:009570  | abnormal right lung morphology                                           | 7         |
| MP:0003078 | aphasis                                                                  | 7         |
| MP:0003584 | bifid ureter                                                             | 7         |
| MP:0013949 | fusion of axis and occipital bones                                       | 7         |
| MP:0013869 | retropharyngeal edema                                                    | 7         |
| MP:0013847 | retropleural edema                                                       | 7         |
| MP:000153  | rib bifurcation                                                          | 7         |
| MP:002191  | abnormal artery morphology                                               | 6         |
| MP:000079  | abnormal basioccipital bone morphology                                   | 6         |
| MP:000788  | abnormal cerebral cortex morphology                                      | 6         |
| MP:0013995 | abnormal external carotid artery origin                                  | 6         |
| MP:0013845 | abnormal eye muscle topology                                             | 6         |
| MP:002858  | abnormal posterior semicircular canal morphology                          | 6         |
| MP:000759  | abnormal skeletal muscle morphology                                      | 6         |
| MP:0013871 | abnormal stapedial artery topology                                       | 6         |
| MP:001146  | abnormal testis morphology                                               | 6         |
| MP:000681  | abnormal thyroid gland morphology                                        | 6         |
| MP:0004999 | abnormal vertebral arch morphology                                       | 6         |
| MP:0013996 | abnormal vertebral artery origin                                         | 6         |
| MP:0013849 | absent abducens nerve                                                    | 6         |
| MP:0000520 | absent kidney                                                            | 6         |
| MP:0009725 | absent lens vesicle                                                      | 6         |
| MP:0006093 | arteriovenous malformation                                               | 6         |
| MP:0010412 | atrioventricular septal defect                                           | 6         |
| MP:0013932 | fragmented Meckel's cartilage                                            | 6         |
| MP:000963  | fused dorsal root ganglion                                               | 6         |
| MP:005157  | holoprosencephaly                                                       | 6         |
| MP:000480  | increased rib number                                                     | 6         |
| MP:0013922 | persistent dorsal ophthalmic artery                                      | 6         |
| MP:0013652 | retro-esophageal left subclavian artery                                   | 6         |
| MP:0004160 | retroesophageal right subclavian artery                                   | 6         |
| MP:0002801 | short tongue                                                             | 6         |
| MP:0002989 | small kidney                                                             | 6         |
| MP:0013852 | abnormal Mullerian duct topology                                         | 5         |
| MP:0010595 | abnormal aortic valve cusp morphology                                    | 5         |
| MP:000297  | abnormal atrioventricular cushion morphology                             | 5         |
| MP:0013186 | abnormal basilar artery morphology                                       | 5         |
| MP:0002152 | abnormal brain morphology                                                | 5         |
| MP:0013874 | abnormal ductus venosus topology                                         | 5         |
| MP:0013945 | abnormal elbow joint morphology                                          | 5         |
| MP:0000559 | abnormal femur morphology                                                | 5         |
| MP:0006063 | abnormal inferior vena cava morphology                                  | 5         |
| MP:0002135 | abnormal kidney morphology                                               | 5         |
| MP:0001879 | abnormal lymphatic vessel morphology                                     | 5         |
| MP:0005236 | abnormal olfactory nerve morphology                                      | 5         |
| MP:000150  | abnormal rib morphology                                                  | 5         |
| MP:0004539 | absent maxilla                                                           | 5         |
| MP:0003451 | absent olfactory bulb                                                    | 5         |
| MP:0001014 | absent superior cervical ganglion                                        | 5         |
| MP:0014003 | additional anastomosis between intracranial vertebral arteries           | 5         |
| MP:0012548 | myelocèle                                                                | 5         |
| MP:0000273 | overriding aortic valve                                                  | 5         |
| MP:0000964 | small dorsal root ganglion                                               | 5         |
| MP:0000964 | spleen hypoplasia                                                        | 5         |
| MP:0013928 | thin motoric part of trigeminal nerve                                    | 5         |
| MP ID       | MP term                                                                 | Frequency |
|------------|--------------------------------------------------------------------------|-----------|
| MP:0002199 | abnormal brain commissure morphology                                      | 4         |
| MP:0006065 | abnormal heart position or orientation                                     | 4         |
| MP:0002249 | abnormal larynx morphology                                                | 4         |
| MP:0009820 | abnormal liver vasculature morphology                                     | 4         |
| MP:0005105 | abnormal middle ear ossicle morphology                                    | 4         |
| MP:0004164 | abnormal neurohypophysis morphology                                      | 4         |
| MP:0013994 | abnormal parasellar internal carotid artery branch morphology             | 4         |
| MP:0006065 | abnormal heart position or orientation                                     | 4         |
| MP:0009820 | abnormal liver vasculature morphology                                     | 4         |
| MP:0005105 | abnormal middle ear ossicle morphology                                    | 4         |
| MP:0011655 | abnormal systemic artery morphology                                       | 4         |
| MP:0011513 | abnormal vertebral artery morphology                                      | 4         |
| MP:0013855 | absent celiac artery                                                      | 4         |
| MP:0013833 | absent olfactory nerve                                                    | 4         |
| MP:0013362 | absent pineal gland                                                       | 4         |
| MP:0014006 | absent posterior communicating artery                                      | 4         |
| MP:0013913 | absent rib-vertebral column attachment                                    | 4         |
| MP:0004846 | absent skeletal muscle                                                    | 4         |
| MP:0013855 | abnormal brain white matter morphology                                    | 3         |
| MP:0006065 | abnormal cervical atlas morphology                                         | 3         |
| MP:0000820 | abnormal choroid atlas morphology                                         | 3         |
| MP:0013873 | abnormal ductus venosus morphology                                        | 3         |
| MP:0010439 | abnormal hepatic vein morphology                                          | 3         |
| MP:0000823 | abnormal lateral hepatic vein morphology                                  | 3         |
| MP:0000598 | abnormal liver morphology                                                 | 3         |
| MP:0000897 | abnormal midbrain morphology                                              | 3         |
| MP:0013861 | abnormal pancreas topology                                                | 3         |
| MP:000613  | abnormal salivary gland morphology                                        | 3         |
| MP:0013943 | abnormal ureter topology                                                  | 3         |
| MP:001100  | abnormal vagus ganglion morphology                                        | 3         |
| MP:0014002 | absent extracranial vertebral artery segment                              | 3         |
| MP:0013929 | absent eye muscles                                                        | 3         |
| MP:0003722 | absent vertebral arch                                                     | 3         |
| MP:0010404 | absent neuronal ganglion                                                  | 3         |
| MP:0002199 | abnormal brain white matter morphology                                    | 3         |
| MP:0010439 | abnormal heart position or orientation                                     | 3         |
| MP:0000820 | abnormal choroid atlas morphology                                         | 3         |
| MP:0013873 | abnormal ductus venosus morphology                                        | 3         |
| MP:0010439 | abnormal hepatic vein morphology                                          | 3         |
| MP:0000823 | abnormal lateral hepatic vein morphology                                  | 3         |
| MP:0000598 | abnormal liver morphology                                                 | 3         |
| MP:0000897 | abnormal midbrain morphology                                              | 3         |
| MP:0013861 | abnormal pancreas topology                                                | 3         |
| MP:000613  | abnormal salivary gland morphology                                        | 3         |
| MP:0013943 | abnormal ureter topology                                                  | 3         |
| MP:001100  | abnormal vagus ganglion morphology                                        | 3         |
| MP:0014002 | absent extracranial vertebral artery segment                              | 3         |
| MP:0013929 | absent eye muscles                                                        | 3         |
| MP:0003722 | absent vertebral arch                                                     | 3         |
| MP:0010404 | absent neuronal ganglion                                                  | 3         |
| MP:0002199 | abnormal brain white matter morphology                                    | 3         |
| MP:0010439 | abnormal heart position or orientation                                     | 3         |
| MP:0000820 | abnormal choroid atlas morphology                                         | 3         |
| MP:0013873 | abnormal ductus venosus morphology                                        | 3         |
| MP:0010439 | abnormal hepatic vein morphology                                          | 3         |
| MP:0000823 | abnormal lateral hepatic vein morphology                                  | 3         |
| MP:0000598 | abnormal liver morphology                                                 | 3         |
| MP:0000897 | abnormal midbrain morphology                                              | 3         |
| MP:0013861 | abnormal pancreas topology                                                | 3         |
| MP:000613  | abnormal salivary gland morphology                                        | 3         |
| MP:0013943 | abnormal ureter topology                                                  | 3         |
| MP:001100  | abnormal vagus ganglion morphology                                        | 3         |
| MP:0014002 | absent extracranial vertebral artery segment                              | 3         |
| MP:0013929 | absent eye muscles                                                        | 3         |
| MP:0003722 | absent vertebral arch                                                     | 3         |
| MP:0010404 | absent neuronal ganglion                                                  | 3         |
| MP:0002199 | abnormal brain white matter morphology                                    | 3         |
| MP:0010439 | abnormal heart position or orientation                                     | 3         |
| MP:0000820 | abnormal choroid atlas morphology                                         | 3         |
| MP:0013873 | abnormal ductus venosus morphology                                        | 3         |
| MP:0010439 | abnormal hepatic vein morphology                                          | 3         |
| MP:0000823 | abnormal lateral hepatic vein morphology                                  | 3         |
| MP:0000598 | abnormal liver morphology                                                 | 3         |
| MP:0000897 | abnormal midbrain morphology                                              | 3         |
| MP:0013861 | abnormal pancreas topology                                                | 3         |
| MP:000613  | abnormal salivary gland morphology                                        | 3         |
| MP:0013943 | abnormal ureter topology                                                  | 3         |
| MP:001100  | abnormal vagus ganglion morphology                                        | 3         |
| MP:0014002 | absent extracranial vertebral artery segment                              | 3         |
| MP:0013929 | absent eye muscles                                                        | 3         |
| MP:0003722 | absent vertebral arch                                                     | 3         |
| MP:0010404 | absent neuronal ganglion                                                  | 3         |
| MP:0002199 | abnormal brain white matter morphology                                    | 3         |
| MP:0010439 | abnormal heart position or orientation                                     | 3         |
| MP:0000820 | abnormal choroid atlas morphology                                         | 3         |
| MP:0013873 | abnormal ductus venosus morphology                                        | 3         |
| MP:0010439 | abnormal hepatic vein morphology                                          | 3         |
| MP:0000823 | abnormal lateral hepatic vein morphology                                  | 3         |
| MP:0000598 | abnormal liver morphology                                                 | 3         |
| MP:0000897 | abnormal midbrain morphology                                              | 3         |
| MP:0013861 | abnormal pancreas topology                                                | 3         |
| MP:000613  | abnormal salivary gland morphology                                        | 3         |
| MP:0013943 | abnormal ureter topology                                                  | 3         |
| MP:001100  | abnormal vagus ganglion morphology                                        | 3         |
| MP:0014002 | absent extracranial vertebral artery segment                              | 3         |
| MP:0013929 | absent eye muscles                                                        | 3         |
| MP:0003722 | absent vertebral arch                                                     | 3         |
| MP:0010404 | absent neuronal ganglion                                                  | 3         |
| MP ID    | MP term                                      | Frequency |
|----------|----------------------------------------------|-----------|
| MP:0003130 | anal atresia                                 | 2         |
| MP:0010463 | aorta stenosis                               | 2         |
| MP:0004055 | atrium hypoplasia                            | 2         |
| MP:0010406 | common atrium                                | 2         |
| MP:0003586 | dilated atrium                               | 2         |
| MP:0013981 | double lumen aortic arch                     | 2         |
| MP:0014018 | embryo tumor                                 | 2         |
| MP:0010200 | enlarged lymphatic vessel                     | 2         |
| MP:0008536 | enlarged third ventricle                      | 2         |
| MP:0002015 | epithelioid cysts                            | 2         |
| MP:0004201 | fetal growth retardation                     | 2         |
| MP:0010977 | fused right lung lobes                       | 2         |
| MP:0013982 | inverse situs of great intrathoracic arteries | 2         |
| MP:0010647 | left atrium hypoplasia                       | 2         |
| MP:0000600 | liver hypoplasia                             | 2         |
| MP:0000618 | small salivary gland                         | 2         |
| MP:0010102 | small superior vagus ganglion                | 2         |
| MP:0000706 | small thymus                                 | 2         |
| MP:0011249 | abdominal situs inversus                     | 1         |
| MP:0000639 | abnormal adrenal gland morphology            | 1         |
| MP:0010592 | abnormal atrioventricular septum morphology  | 1         |
| MP:0002745 | abnormal atrioventricular valve morphology    | 1         |
| MP:0001614 | abnormal blood vessel morphology              | 1         |
| MP:0000494 | abnormal cecum morphology                    | 1         |
| MP:0013862 | abnormal cecum position                      | 1         |
| MP:0010744 | abnormal cervical flexure morphology         | 1         |
| MP:0003048 | abnormal cervical vertebrae morphology       | 1         |
| MP:0009495 | abnormal common bile duct morphology         | 1         |
| MP:0012729 | abnormal common carotid artery morphology    | 1         |
| MP:0013930 | abnormal digastric muscle connection         | 1         |
| MP:0004252 | abnormal direction of heart looping          | 1         |
| MP:0014022 | abnormal duodenum topology                   | 1         |
| MP:0013924 | abnormal dural venous sinus morphology       | 1         |
| MP:0013927 | abnormal facial nerve topology               | 1         |
| MP:0006107 | abnormal fetal atrioventricular canal         | 1         |

| MP ID    | MP term                                      | Frequency |
|----------|----------------------------------------------|-----------|
| MP:0013975 | abnormal coronary sinus connection            | 2         |
| MP:0002279 | abnormal diaphragm morphology                | 2         |
| MP:0013815 | abnormal digastric muscle morphology         | 2         |
| MP:0013865 | abnormal dorsal pancreas topology            | 2         |
| MP:0000961 | abnormal dorsal root ganglion morphology     | 2         |
| MP:0013950 | abnormal dorsal root ganglion topology       | 2         |
| MP:0006011 | abnormal endolymphatic duct morphology       | 2         |
| MP:0013918 | abnormal endolymphatic sac topology          | 2         |
| MP:0006033 | abnormal external auditory canal morphology  | 2         |
| MP:000266  | abnormal heart morphology                    | 2         |
| MP:000356  | abnormal hyoid bone morphology               | 2         |
| MP:0013966 | abnormal infrahyoid muscle morphology        | 2         |
| MP:000489  | abnormal large intestine morphology          | 2         |
| MP:008986  | abnormal liver parenchyma morphology         | 2         |
| MP:001175  | abnormal lung morphology                     | 2         |
| MP:000458  | abnormal mandible morphology                 | 2         |
| MP:003632  | abnormal nervous system morphology           | 2         |
| MP:001330  | abnormal optic nerve morphology              | 2         |
| MP:002177  | abnormal outer ear morphology                | 2         |
| MP:000492  | abnormal rectum morphology                   | 2         |
| MP:002428  | abnormal semicircular canal morphology       | 2         |
| MP:002746  | abnormal semilunar valve morphology          | 2         |
| MP:000496  | abnormal small intestine morphology          | 2         |
| MP:005107  | abnormal stapes morphology                   | 2         |
| MP:003230  | abnormal umbilical artery morphology         | 2         |
| MP:002725  | abnormal vein morphology                     | 2         |
| MP:009707  | absent external auditory canal               | 2         |
| MP:0013987 | absent intrahepatic inferior vena cava segment | 2         |
| MP:0009771 | absent optic chiasm                          | 2         |
| MP:0013999 | absent parasellar internal carotid artery    | 2         |
| MP:0013809 | absent pectinate muscle                      | 2         |
| MP:004571  | absent vagus nerve                           | 2         |
| MP:000140  | absent vertebral pedicles                    | 2         |
| MP ID    | MP term                                      | Frequency |
|----------|----------------------------------------------|-----------|
| MP:0000828 | abnormal fourth ventricle morphology         | 1         |
| MP:0005272 | abnormal temporal bone morphology            | 1         |
| MP:000826  | abnormal third ventricle morphology         | 1         |
| MP:0013935 | abnormal thyroid capsule morphology         | 1         |
| MP:002282  | abnormal trachea morphology                 | 1         |
| MP:001065  | abnormal trigeminal nerve morphology        | 1         |
| MP:0010667 | abnormal umbilical vein morphology          | 1         |
| MP:000534  | abnormal ureter morphology                  | 1         |
| MP:0013925 | abnormal vascular plexus formation          | 1         |
| MP:000137  | abnormal vertebrae morphology               | 1         |
| MP:000787  | abnormal telencephalon morphology           | 1         |
| MP:0005084 | abnormal gallbladder morphology             | 1         |
| MP:0013105 | abnormal heart atrium morphology            | 1         |
| MP:00003922| abnormal right atrium morphology            | 1         |
| MP:0013814 | abnormal hepatic portal vein connection     | 1         |
| MP:0013853 | abnormal hepatic portal vein formation      | 1         |
| MP:000324  | abnormal hepatic portal vein morphology     | 1         |
| MP:0009804 | abnormal hepatic vein connection            | 1         |
| MP:000926  | abnormal humerus morphology                 | 1         |
| MP:0000013 | abnormal hyoid bone greater horn morphology | 1         |
| MP:0009913 | abnormal heart atrium morphology            | 1         |
| MP:0013824 | abnormal hypoglossal canal morphology       | 1         |
| MP:0002859 | abnormal inner ear canal fusion             | 1         |
| MP:0000148 | abnormal intraventricular foramen morphology| 1         |
| MP:0000281 | abnormal intraventricular septum morphology | 1         |
| MP:0000475 | abnormal left ventricle superior connection  | 1         |
| MP:000481  | abnormal lung size                          | 1         |
| MP:0013841 | abnormal lymphatic vessel topology          | 1         |
| MP:0003792 | abnormal major salivary gland morphology    | 1         |
| MP:0000455 | abnormal maxilla morphology                 | 1         |
| MP:0000452 | abnormal mouth morphology                   | 1         |
| MP:0002108 | abnormal muscle morphology                  | 1         |
| MP:0004056 | abnormal myocardium compact layer morphology| 1         |
| MP:0005269 | abnormal occipital bone morphology          | 1         |
| MP:0013815 | abnormal oral cavity morphology             | 1         |
| MP:0014011 | abnormal ovary tissue architecture          | 1         |
| MP:0000459 | abnormal pelvic girdle bone morphology      | 1         |
| MP:0002778 | abnormal pulmonary valve morphology         | 1         |
| MP:0009571 | abnormal right lung accessory lobe morphology| 1         |
| MP:0009688 | abnormal spinal cord central canal morphology| 1         |
| MP:0005323 | abnormal styloid process morphology        | 1         |
| MP:0013975 | abnormal subclavian artery origin           | 1         |
| MP:0001011 | abnormal superior cervical ganglion morphology| 1         |
| MP:0000787 | abnormal telencephalon morphology           | 1         |
| MP:0005272 | abnormal temporal bone morphology           | 1         |
| MP:0000826 | abnormal third ventricle morphology        | 1         |
| MP:0002368 | abnormal thymus capsule morphology         | 1         |
| MP:0002282 | abnormal trachea morphology                 | 1         |
| MP:0001065 | abnormal trigeminal nerve morphology        | 1         |
| MP:0013925 | abnormal vascular plexus formation          | 1         |
| MP:0000137 | abnormal vertebrae morphology               | 1         |
| MP:0005274 | abnormal viscerocranium morphology         | 1         |
| MP:0010666 | abnormal vitelline vein morphology          | 1         |
| MP:0014004 | absent basilar artery segment               | 1         |
| MP:0008129 | absent brain internal capsule              | 1         |
| MP:0013998 | absent canicular internal carotid artery segment| 1         |
| MP:0008400 | absent dorsal root ganglion                 | 1         |
| MP:0013880 | absent ductus venosus                       | 1         |
| MP:0013914 | absent intracranial segment of vertebral artery| 1         |
| MP:0013937 | absent lobe of thyroid gland                | 1         |
| MP:0000629 | absent mammary gland                        | 1         |
| MP:0013926 | absent neurohypophysis                      | 1         |
| MP:0013988 | absent portal vein segment                  | 1         |
| MP:0000050 | absent posterior commisure                  | 1         |
| MP:0000614 | absent salivary gland                       | 1         |
| MP:0001382 | absent segment of anterior cerebral artery  | 1         |
| MP:0000690 | absent spleen                               | 1         |
| MP:0008386 | absent styloid process                      | 1         |
| MP:0002728 | absent tibia                                | 1         |
| MP:0009905 | absent tongue                               | 1         |
| MP:0001064 | absent trochlear nerve                      | 1         |
| MP:0013959 | absent vomeronasal organ                    | 1         |
| MP:0013860 | anastomosis between common carotid and vertebral artery | 1 |
| MP ID     | MP term                              | Frequency |
|-----------|--------------------------------------|-----------|
| MP:0013935 | basal brain tissue herniation         | 1         |
| MP:0010527 | bicuspid pulmonary valve              | 1         |
| MP:0011797 | blind ureter                          | 1         |
| MP:0010607 | common atrioventricular valve         | 1         |
| MP:0004686 | decreased length of long bones        | 1         |
| MP:0009532 | decreased parotid gland size          | 1         |
| MP:0004648 | decreased thoracic vertebrae number   | 1         |
| MP:0011965 | decreased total retina thickness      | 1         |
| MP:0001247 | dermal cysts                          | 1         |
| MP:0000825 | dilated lateral ventricles            | 1         |
| MP:0009144 | dilated pancreatic duct              | 1         |
| MP:0004938 | dilated vasculature                   | 1         |
| MP:0011380 | enlarged brain ventricles             | 1         |
| MP:0013864 | enlarged paraumbilical vein           | 1         |
| MP:0003595 | epididymal cyst                       | 1         |
| MP:0002947 | increased hemangioma incidence        | 1         |
| MP:0001634 | internal hemorrhage                   | 1         |
| MP:0011974 | intestinal stenosis                   | 1         |
| MP:0001916 | intracerebral hemorrhage              | 1         |
| MP:0003178 | left pulmonary isomerism              | 1         |
| MP:0013953 | left sided brachiocephalic trunk      | 1         |
| MP:0003327 | liver cysts                           | 1         |
| MP:0003888 | liver hemorrhage                      | 1         |
| MP:0000162 | lordosis                              | 1         |
| MP:0010854 | lung situs inversus                   | 1         |
| MP:0005287 | narrow eye opening                    | 1         |
| MP:0004442 | occipital bone foramen                | 1         |
| MP:0000565 | oligodactyly                          | 1         |
| MP:0006221 | optic nerve hypoplasia                | 1         |
| MP:0013933 | short Meckel’s cartilage              | 1         |
| MP:0002766 | situs inversus                        | 1         |
| MP:0002768 | small adrenal glands                  | 1         |
| MP:0001306 | small lens                            | 1         |
| MP:0013923 | small prevertebral sympathetic ganglia| 1         |
| MP:0006254 | thin cerebral cortex                  | 1         |
| MP:0013829 | thin splanchnic nerve                 | 1         |
| MP:0013832 | thin vagus nerve                      | 1         |
| MP:0003499 | thyroid hypoplasia                    | 1         |
| MP:0009904 | tongue hypoplasia                     | 1         |
| MP:0011697 | vacuolated lens                       | 1         |
| MP:0013831 | vagus nerve compression               | 1         |
| MP:0004609 | vertebral fusion                      | 1         |

Individual phenotypes show highly variable penetrance

Perhaps the most striking finding of the DMDD study is the almost complete absence of any fully penetrant abnormalities. Amongst lines for which more than a single embryo was analysed, only three phenotypes showed 100% penetrance: abnormal perichondrial ossification (1 line; 10 mutant embryos), small nodose ganglion (1 line; 4 embryos) and small trigeminal ganglion (1 line, 3 embryos). Furthermore, most defects showed surprisingly low penetrance. A penetrance greater than 75% within the line was only found for 7% of detected phenotypes. In contrast, over half (55%) of the scored abnormalities had a penetrance of 25% or less (Table 4). This is graphically illustrated in Figure 5A, in which the scored phenotypes are clustered according to high level MP ontology terms (broadly reflecting distinct organ systems, tissues or body regions) and the prevalence of each in the 42 mutant lines categorised by penetrance. All phenotypes show a broad range of penetrance, about half showing roughly symmetrical distribution of penetrance, with similar numbers of lines both above and below 50%. Interestingly, it is possible also to distinguish several phenotypes where penetrance is noticeably skewed. Abnormalities affecting the cardiovascular system, nervous system and skeleton all affected a relatively large number of lines and each showed a striking bias towards higher penetrance values. A second group of abnormalities encompassing liver/biliary, respiratory, renal and hearing systems showed a converse bias to penetrance values below 50% (Figure 5A).

When grouped into such high level MP ontology terms, the most common group of abnormalities are those affecting the cardiovascular system, examples of which affect embryos in every single mutant line studied. Almost as prevalent are nervous system phenotypes, which are detected in 80% of the lines studied. Re-plotting the data summarised by intermediate level MP term slim provides a more detailed view of the prevalence and variability in penetrance of phenotypes (Figure 5B). At this level of resolution, for example, cardiovascular defects are subdivided into two broad categories; those encompassing abnormalities in blood vessel morphology or topology ("abnormal blood vessel morphology" and most phenotypes within "abnormal cardiovascular development") and those affecting the heart and its great vessels ("abnormal heart morphology"). Viewed in this way, it is clear that detection of cardiovascular defects in all lines examined results from the presence of phenotypes in the vasculature. These range from relatively major defects such as absence of the ductus venosus, interrupted aortic arch or arterial stenosis, to more minor alterations in vascular topology in different regions of the embryo. Cardiac abnormalities nevertheless remain prevalent, affecting almost two thirds (27/42) of the showing abnormal topology or unusual thinness of the nerve (13 and 9 lines respectively). Overall, scored phenotypes affected all the major organ systems at E14.5 (Figure 5A) and multiple organs or tissues were frequently affected within individual embryos, or collectively within a mutant line (Figure 2 and Supplementary Figure 2 and Supplementary Figure 3). The complete listing of scored phenotypes is presented in Supplementary Table 4, organised according to the MP ontology slims adopted by the DMDD, with data ranked according to prevalence in mutant lines.
Multiple abnormalities are evident in homozygous mutant embryos. The Mammalian Phenotype Ontology terms scored for (A) each embryo, and (B) each line were normalised to remove duplicate ontology terms. The number of distinct phenotypes scored that fell into categories with a window width of 10 were plotted to show the total number of embryos and lines respectively in each category.
Table 2B. Frequency of phenotypes identified in wild type embryos. The Mammalian Phenotype Ontology terms describing phenotypes observed in each embryo were normalised to remove duplicates and the list then ranked in descending order by frequency of embryos exhibiting each phenotype.

| MP ID       | MP term                                               | Frequency |
|-------------|-------------------------------------------------------|-----------|
| MP:0002169  | no abnormal phenotype detected                       | 78        |
| MP:0013971  | blood in lymph vessels                                | 5         |
| MP:0011493  | double ureter                                        | 4         |
| MP:0013852  | abnormal Mullerian duct topology                     | 3         |
| MP:0000783  | abnormal forebrain morphology                         | 3         |
| MP:0013876  | absent ductus venosus valve                           | 3         |
| MP:0013840  | absent segment of posterior cerebral artery           | 3         |
| MP:0011803  | double kidney pelvis                                  | 3         |
| MP:0003826  | abnormal Mullerian duct morphology                   | 2         |
| MP:0013877  | abnormal ductus venosus valve morphology              | 2         |
| MP:0006063  | abnormal inferior vena cava morphology               | 2         |
| MP:0014003  | additional anastomosis between intracranial vertebral arteries | 2         |
| MP:0003586  | diliated ureter                                       | 2         |
| MP:0011683  | dual inferior vena cava                               | 2         |

Table 3. Overlap of identified phenotypes between homozygous mutant and wild type embryos within each line. Mutant lines showing a phenotype shared by at least one homozygous mutant and one wild type embryo are listed, along with the MP term, its MP ID and it penetrance amongst the mutant and wildtype embryos. For each line where an overlap is identified, the ratio of shared phenotypes to the total number of unique phenotypes identified in mutant embryos is also presented.

| Allele                                  | Phenotypes shared by homozygous mutants and wild type embryos | MP ID | Penetrance in mutants | Penetrance in wild types | Ratio of shared: total mutant phenotypes |
|-----------------------------------------|--------------------------------------------------------------|-------|-----------------------|--------------------------|------------------------------------------|
| Adamts3<tm1b(KOMP)Wtsi>                | abnormal forebrain morphology                               | MP:000783 | 2/7                   | 1/3                      | 2/44                                     |
| Adcy9<tm1b(EUCOMM)Wtsi>                | abnormal Mullerian duct topology                            | MP:0013852 | 1/7                   | 1/3                      |                                          |
| Celf4<tm1a(EUCOMM)Wtsi>                | blood in lymph vessels                                     | MP:003826 | 1/8                   | 1/3                      | 2/20                                     |
| Chtho<tm1a(EUCOMM)Wtsi>                | abnormal Mullerian duct morphology                         | MP:0013971 | 1/8                   | 1/3                      |                                          |
| Cir1<tm3a(KOMP)Wtsi>                   | additional anastomosis between intracranial vertebral arteries | MP:0014003 | 1/3                   | 1/2                      | 1/29                                     |
| Nsun2<tm1a(EUCOMM)Wtsi>                | absent ductus venosus valve                                 | MP:0013876 | 1/6                   | 1/2                      | 1/37                                     |
| Pspht<tm1a(EUCOMM)Hmgu>                | blood in lymph vessels                                     | MP:0013971 | 1/8                   | 1/3                      | 1/109                                    |
| Tcf7l2<tm1a(EUCOMM)Wtsi>               | absent ductus venosus valve                                 | MP:0013876 | 2/5                   | 1/4                      | 3/32                                     |
|                                              | enlarged liver sinusoidal spaces                            | MP:0000602 | 2/5                   | 1/4                      |                                          |
|                                              | abnormal eye muscle morphology                              | MP:0003686 | 3/5                   | 1/4                      |                                          |
| Traf6<tm2a(EUCOMM)Wtsi>                | blood in lymph vessels                                     | MP:0013971 | 4/9                   | 1/5                      | 1/39                                     |
| Unk<tm1a(KOMP)Wtsi>                    | absent ureter                                              | MP:0003722 | 2/5                   | 1/2                      | 2/10                                     |
|                                              | absent kidney                                              | MP:000520 | 2/5                   | 1/2                      |                                          |
mutant lines. These encompass malformations in all regions of the four-chambered heart and its great vessels, including both atrial and ventricular septal defects, atrioventricular septal defects, common arterial trunk, double outlet right ventricle, transposition of the great arteries, bicuspid aortic valve, common truncal valve and abnormally thin myocardium. After blood vessel and cardiac abnormalities, the third most prevalent group of phenotypes detected were those affecting brain morphology (Figure 5B), most commonly the forebrain (Figure 6 and Supplementary Table 1A).

In order to assess the relative significance of each phenotype in the context of variable penetrance, we re-examined their ranking distribution after weighting each phenotype according to its individual prevalence. This provides a plot of cumulative line penetrance for each of the 70 intermediate level MP term slim (Figure 7). Whilst abnormalities in blood vessel morphology and structure of the heart remain amongst the most prevalent phenotypes, weighting by penetrance has a significant impact on the ranking of other phenotypes. Notably, the relative ranking of “abnormal brain morphology” and “abnormal somatic nervous system morphology” is increased, with both now lying in the five most prevalent abnormalities scored. This change is largely driven by the relatively high prevalence associated with abnormalities in forebrain morphology and hypoglossal nerve structure or presence, respectively.

Phenotype penetrance is affected by allele type
Of the 42 mutant lines studied, 22 contained the tm1a insertion allele, compared with 20 containing exon deletions (19 tm1b and 1 CRISPR). With either group, blood vessel, heart and brain morphology remain amongst the most commonly observed.
Figure 3. Examples of frequently observed abnormalities in mutant embryos. A–C. Subcutaneous edema. Original HREM sections showing a massive (asterisk) (A), mild (B), and unilaterally located subcutaneous edema (C). Note the shrinkage artefacts in B and C, which complicate post mortem diagnosis. D–F. Perimembranous septal defect. Normal situation in a control (D) as appearing in an original HREM section. Defect (asterisk) as appearing in an original HREM section (E) and a 3D volume model (F). G–I. Fusion of vertebral arches. Normal situation in a control (G) as appearing in a sagittal section. Fused articular processes (arrowheads) of subsequent vertebrae in a sagittal (H) and a coronal section (I). J–L. Abnormal eye muscle morphology as appearing in original HREM sections. Normal situation in a control (J). Thinning of the lateral rectus muscle (lrm) (K). Absence of the lateral rectus muscle (lrm) (L). da, descending aorta; e, esophagus; g, adrenal gland; hb, hyoid bone; i, intestine; k, kidney; l, lung; la, left atrium; le, lens; li, liver; lrm, lateral rectus muscle; lv, left ventricle; lv, larynx; mrm, medial rectus muscle; oc, optic cup; on, optic nerve; ra, right atrium; rv, right ventricle; sc, spinal chord; t, tongue; tr, trachea; v, body of vertebra; va, arch of vertebra. Scale bars: 1 mm.
Figure 4. Other frequently observed abnormalities in mutant embryos. A and B. Abnormal hypoglossal nerve in original HREM sections through the head of Prc2b<sup>−/−</sup> (A) and a Polb<sup>−/−</sup> (B) embryo. Note the missing right hypoglossal nerve (arrowhead, inset) in A and the thinning of both hypoglossal nerves (hn) in B, C–E. Abnormalities that also occur in controls. Persisting craniopharyngeal duct (arrowhead) as appearing in sagittal sections (C). Split tip of tail featured by volume models (D) and vesicles (arrowheads) in the lens (le) as appearing in an original HREM section (E).

abnormalities. There is however a clear difference in phenotype penetrance between the two groups: phenotypes are significantly less penetrant with tm1a alleles (compare Figure 5B with Figure 8A and B).

Phenotyping embryos required new MP terms
Adoption of a formal, standardised ontology for scoring abnormalities provides an essential framework for analysing the data and facilitating structured search enquiries. However, during the course of the DMDD programme and its pilot study<sup>9</sup>, it became clear that additional terms were required in order to adequately describe abnormalities in embryo, as opposed to adult structures. A further outcome of the DMDD study has therefore been the creation of 142 new MP terms to accommodate the range of abnormalities we have observed (Table 7). These include, for example, thin motoric part of the trigeminal nerve (MP:0013928; http://www.ontobee.org/ontology/MP?iri=http://purl.obolibrary.org/obo/MP_0013928), blood in lymph vessels (MP:0013971; http://www.ontobee.org/ontology/MP?iri=http://purl.obolibrary.org/obo/MP_0013971), double lumen aortic arch (MP:0013981; http://www.ontobee.org/ontology/MP?iri=http://purl.obolibrary.org/obo/MP_0013981), abnormal elbow joint morphology (MP:0013945; http://www.ontobee.org/ontology/MP?iri=http://purl.obolibrary.org/obo/MP_0013945), and intramural bleeding in blood vessel wall (MP:0014020; http://www.ontobee.org/ontology/MP?iri=http://purl.obolibrary.org/obo/MP_0014020) (Figure 9).

Discussion
Since approximately one third of gene knockouts in the mouse prove to be embryonic or perinatal lethal<sup>1–3</sup>, further study of such lines offers a unique opportunity to better understand the genetic regulation of embryo development and identify genetic determinants of congenital abnormalities. The data accumulated during three years of the DMDD programme provide the first opportunity to study in detail the identity, range and prevalence of morphological abnormalities in such mutants and offer a window on the opportunities (and pitfalls) such systematic studies present.

The current analysis is restricted to a single developmental stage (E14.5) when most organ systems of the embryo have developed their definitive fetal appearance and the body plan is broadly similar to that of the adult mouse. Whilst this provides obvious practical advantages for a systematic, high throughput phenotyping programme, it is of course an arbitrary choice with respect to the time course of individual gene function and the consequences of gene ablation. Indeed, about 60% of the lethal lines entering
Figure 5. Variable prevalence and penetrance of individual phenotypes in mutant embryos. Data from the global analysis of the frequency of phenotype terms (see Materials and Methods) was plotted to show the number of lines falling into each of the observed phenotype categories. The colours indicate the number of lines falling into each of the distinct penetrance categories. The data was ordered according to line frequency, and subsequently by the numbers seen in the penetrance categories. (A) shows the phenotype annotations summarised using the high level DMDD ontology slim, (B) shows the phenotype annotations summarised using the intermediate level DMDD ontology slim.
the DMDD pipeline fail to provide homozygous mutant offspring by E14.5, with half of those causing lethality prior to E9.5 [see also 2]. The data here therefore comes from a subset of lethal lines. Furthermore, phenotypes observed at a single time point most likely combine more immediate consequences of individual gene loss with more distant or secondary consequences. Tearing out the role of regulative or compensatory changes from primary effects of gene loss is likely to be difficult. Despite these caveats, there are, nevertheless, several striking findings that emerge from detailed phenotype analysis.

Our finding that some manifestation of edema (generally subcutaneous) is the most common phenotype could indicate an unappreciated complexity in the genetic controls regulating fluid balance or tissue integrity of vascular or lymphatic components. Edema may also represent a common outcome for a wide range of pathophysiological perturbations, as has been proposed for the association of non-immune hydrops fetalis with human fetal loss [11,12]. The prevalence of cardiovascular defects is also consistent with the well established finding that cardiac abnormalities are the most common congenital defect in human newborns [13]. Some caution is necessary in considering the mouse data, since as we have shown, a significant proportion of cardiovascular phenotypes comprise apparently minor alterations in blood vessel topology, the impact of which on normal development remains unclear. However, in addition to these, the lines we have studied show a range of severe abnormalities in cardiac structure that are both relatively prevalent and mirror the range of congenital abnormalities seen in humans. Despite the largely random selection of genes studied in screens such as DMDD, their identification as embryonic lethal therefore provides a dramatic enrichment for potential cardiac developmental disease alleles.

Phenotypes affecting neural tissue also prove to be relatively prevalent in mutant embryos. We are limited in the present analysis to identifying a subset of neural deficits readily identified from HREM imaging. This restricts identifiable phenotypes to relatively gross alterations in brain and neural tube morphology, or changes affecting major nerves. Amongst the latter, the frequency with which abnormalities affecting the hypoglossal nerve have been detected is perhaps not so surprising, since these (like abnormalities detected in the motoric portion of the trigeminal nerve) may compromise suckling and lead to perinatal lethality.

The multiplicity of phenotypes frequently detected in individual mutant embryos is not unexpected, given the nature of a single time point screening procedure, combined with the likely pleiotropic effects of individual gene loss. However, the most striking and surprising finding to emerge from the DMDD phenotype data is that virtually all phenotypes are incompletely (and frequently poorly) penetrant, despite the use of the isogenic C57BL/6N mouse strain. Combined with the observation of overlapping but distinct spectra of phenotypes between individual embryos from a single

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**Table 4. Variability in mutant phenotype penetrance.**

Every distinct phenotype scored in each line was listed along with its penetrance (i.e. the number of embryos showing the phenotype divided by the total number of embryos analysed for that line). Scored phenotypes were then ranked by penetrance value to obtain the proportions falling within the four ranges shown. (Note that all data from the lines Otud7b, Npat and Dnx5 were removed from the analysis, since in each case, these were obtained from examination of a single embryo).

| Penetration range | Phenotypes scored (homozygous mutants) | % |
|-------------------|----------------------------------------|---|
| <25%              | 673                                    | 55.21% |
| 26–50%            | 343                                    | 28.14% |
| 51–75%            | 118                                    | 9.68%  |
| >75%              | 85                                     | 6.97%  |

**Table 5. High level MP ontology slim used by DMDD.**

A list of the Mammalian Phenotype Ontology IDs and names of terms selected as the high level ontology slim.

| MP:0002169          | no abnormal phenotype detected          |
| MP:0005375          | adipose tissue phenotype                |
| MP:0005386          | behavior/neurological phenotype         |
| MP:0005385          | cardiovascular system phenotype         |
| MP:0005384          | cellular phenotype                      |
| MP:0005382          | craniofacial phenotype                  |
| MP:0005381          | digestive/alloimentary phenotype        |
| MP:0005380          | embryogenesis phenotype                 |
| MP:0005379          | endocrine/exocrine gland phenotype      |
| MP:0005378          | growth/size/body region phenotype       |
| MP:0005377          | hearing/vestibular/ear phenotype        |
| MP:0005397          | hematopoietic system phenotype          |
| MP:0005376          | homeostasis/metabolism phenotype        |
| MP:0005387          | immune system phenotype                 |
| MP:0010771          | integument phenotype                    |
| MP:0005371          | limbs/digits/tail phenotype             |
| MP:0005370          | liver/biliary system phenotype          |
| MP:0010768          | mortality/aging                         |
| MP:0005369          | muscle phenotype                        |
| MP:0003631          | nervous system phenotype                |
| MP:0001186          | pigmentation phenotype                  |
| MP:0005367          | renal/urinary system phenotype          |
| MP:0005389          | reproductive system phenotype           |
| MP:0005388          | respiratory system phenotype            |
| MP:0005390          | skeleton phenotype                      |
| MP:0005394          | taste/olfact action phenotype           |
| MP:0002006          | tumorogenesis                           |
| MP:0005391          | vision/eye phenotype                    |
line, these findings are challenging to understand, and at a minimum point towards unknown stochastic components affecting
the etiology of each phenotype or the compensatory responses
they elicit. They also demonstrate that efforts to identify linkage
between mouse embryo phenotypes and human developmental
disease are likely to require sophisticated bioinformatic analysis
beyond the obvious issues raised by species differences in anatomy
and physiology.

The observation of a small number of phenotypes amongst the wild
type litter mates of the homozygous mutants raises the important
question: why are phenotypes detected in genetically wild type
embryos? We think there are several possible explanations. One
possibility is that the C57BL/6N mouse strain used for engineer-
ing knockout lines carries a “background load” of abnormalities,
previously unappreciated. Ours is the first systematic study on suffi-
ciently large scale and employing sufficiently high-resolution imag-
ing to detect such abnormalities. None of the phenotypes we have
identified show a high penetrance across both mutants and wild
types of a mutant line and do not therefore suggest themselves as
strain-specific abnormalities. Another possible explanation is that
abnormalities arise as a consequence of de novo mutation. Lastly, at
least with the less profound abnormalities, it is possible that some
phenotypes may prove to be outliers on spectrum of normal mor-pho-
logical variation and should not be considered genuine abnormali-
ties. This highlights an important issue confronting phenotyping
studies: the dearth of large-scale and systematic studies examining
normal embryo morphology that can set a reliable benchmark for
distinguishing abnormalities from normal variation. In this light,
phenotype data may need revision as cumulative experience with
the C57BL/6N and other mouse strains improves our ability to
distinguish abnormalities from normal variation amongst wild
types.

Our study has identified a small number of apparent abnormali-
ties common to both homozygous mutant embryos and wild-
type controls from the C57BL/6N mouse strain and which have
therefore been excluded from the phenotyping procedure. These
include splitting of the tail tip, persistence of the craniopharyngeal
duct with associated fenestration of head bones and the presence
of vesicles in the lens of the eye (Figure 4, panels C–E). Apart from
these, our data offers no clear evidence for other “background”
phenotypes associated with either the C57BL/6N genetic
background or with individual mutant lines. Overall, we
consider that neither the frequency, prevalence nor nature of the
phenotypes identified in wild type embryos impact significantly on

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Figure 6. Abnormal brain morphology phenotypes. A and B. Tissue protrusion (pr) into the 3rd ventricle (III) in an original HREM-section (A) and a volume model (B). Inlay in B shows normal situation in a control. C. Irregular tissue protrusions (arrowheads) on the brain surface in a 4933434E20Rik−/− embryo. D. Abnormal tissue (arrowhead) at the cortex near the lateral sulcus in a Polb−/− embryo. E. Abnormal frontal wall of the lateral ventricles in a H13− embryo. F. Abnormal morphology and tissue architecture (arrowhead) of the frontal forebrain in a Chtop− embryo. G. Abnormal morphology of the wall of the 3rd ventricle and protrusions (arrowhead) on the surface of the diencephalon in a Brd2− embryo. ah, adenohypophysis; f, forebrain; h, hindbrain; ie, inner ear; oc, optic cup; pr, tissue protrusion; tg, trigeminal ganglion; III, 3rd ventricle; Scale bars 1 mm.
Figure 7. Cumulative penetrance of individual phenotypes in mutant embryos. Data from the global analysis of the frequency of phenotype terms (see Materials and Methods) was plotted to show the cumulative penetrance score for each of the phenotype categories observed (i.e. the overall sum of the penetrance scores recorded for the lines showing the phenotype). The Mammalian Phenotype Ontology terms assigned during embryo phenotyping were summarised using the intermediate level DMDD ontology slim, and the data was ordered according to the cumulative penetrance score. The colours indicate the contribution of lines falling into each of the distinct penetrance categories to the cumulative penetrance score.
Figure 8. Influence of allele type on prevalence and penetrance of individual phenotypes in mutant embryos. Data from the global analysis of the frequency of phenotype terms shown in Figure 5A was subdivided by allele type to compare tm1a (Figure 8A) and tm1b (Figure 8B) alleles. Data is summarised using the intermediate level ontology slim and colours indicate the number of lines falling into each of the distinct penetrance categories. The data was ordered according to line frequency and subsequently by numbers seen in the penetrance categories.
### Table 6. Intermediate level MP ontology slim used by DMDD.

A list of the Mammalian Phenotype Ontology IDs and names of terms selected as the intermediate level ontology slim.

| MP:0000001 | mammalian phenotype |
| MP:0002873 | normal phenotype |
| MP:0002169 | no abnormal phenotype detected |
| MP:0005375 | adipose tissue phenotype |
| MP:0000003 | abnormal adipose tissue morphology |
| MP:0005666 | abnormal adipose tissue physiology |
| MP:0004924 | abnormal behavior |
| MP:0020222 | abnormal alertness |
| MP:0011275 | abnormal behavioral response to light |
| MP:0009745 | abnormal behavioral response to xenobiotic |
| MP:0001502 | abnormal circadian rhythm |
| MP:0002069 | abnormal consumption behavior |
| MP:0002572 | abnormal emotion/affect behavior |
| MP:0001440 | abnormal grooming behavior |
| MP:0010698 | abnormal impulsive behavior control |
| MP:0002063 | abnormal learning/memory/conditioning |
| MP:0002066 | abnormal motor capabilities/coordination/movement |
| MP:0002067 | abnormal sensory capabilities/reflexes/nociception |
| MP:0011396 | abnormal sleep behavior |
| MP:0002557 | abnormal social/conspecific interaction |
| MP:0011275 | abnormal social behavior |
| MP:0011275 | abnormal social/conspecific interaction |
| MP:0001529 | abnormal vocalization |
| MP:002822 | catalepsy |
| MP:0002899 | fatigue |
| MP:0002064 | seizures |
| MP:0002127 | abnormal cardiovascular system morphology |
| MP:0001614 | abnormal blood vessel morphology |
| MP:0002925 | abnormal cardiovascular development |
| MP:0000266 | abnormal heart morphology |
| MP:0003279 | aneurysm |
| MP:0013332 | peliosis |
| MP:0001544 | abnormal cardiovascular system physiology |
| MP:0002128 | abnormal blood circulation |
| MP:0010695 | abnormal blood pressure regulation |
| MP:000249 | abnormal blood vessel physiology |
| MP:0004039 | abnormal cardiac cell glucose uptake |
| MP:0002972 | abnormal cardiac muscle contractility |
| MP:0004084 | abnormal cardiac muscle relaxation |
| MP:0011926 | abnormal cardiac valve physiology |
| MP:0011390 | abnormal fetal cardiomyocyte physiology |
| MP:0011925 | abnormal heart echocardiography feature |
| MP:0008775 | abnormal heart ventricle pressure |
| MP:0004085 | abnormal heartbeat |
| MP:0003137 | abnormal impulse conducting system conduction |
| MP:0020095 | abnormal mean heart rate adaptation |
| MP:0004215 | abnormal myocardial fiber physiology |
| MP:003547 | abnormal pulmonary pressure |
| MP:0020092 | abnormal susceptibility to aortic cartilaginous metaplasia |
| MP:0020098 | abnormal susceptibility to diet-induced aortic fatty streak lesions |
| MP:000230 | abnormal systemic arterial blood pressure |
| MP:0004484 | altered response of heart to induced stress |
| MP:0000343 | altered response to myocardial infarction |
| MP:0005330 | cardiomyopathy |
| MP:0001529 | congestive heart failure |
| MP:0001853 | heart inflammation |
| MP:0003328 | portal hypertension |
| MP:0005384 | cellular phenotype |
| MP:0000358 | abnormal cell morphology |
| MP:0005621 | abnormal cell physiology |
| MP:0013258 | abnormal extracellular matrix morphology |
| MP:0003121 | genetic imprinting |
| MP:0005382 | craniofacial phenotype |
| MP:0000428 | abnormal craniofacial morphology |
| MP:0002116 | abnormal craniofacial bone morphology |
| MP:0003935 | abnormal craniofacial development |
| MP:0003743 | abnormal facial morphology |
| MP:0011495 | abnormal head shape |
| MP:0002177 | abnormal outer ear morphology |
| MP:0005381 | abnormal face morphology |
| MP:0000462 | abnormal digestive system morphology |
| MP:0001663 | abnormal digestive system physiology |
| MP:0005380 | embryogenesis phenotype |
| MP:0001672 | abnormal embryogenesis/development |
| MP:0002084 | abnormal developmental patterning |
| MP:0001697 | abnormal embryo size |
| MP:0002085 | abnormal embryonic tissue morphology |
| MP:0008926 | abnormal anterior definitive endoderm morphology |
| MP:00013230 | abnormal cervical sinus morphology |
| MP:0003085 | abnormal egg cylinder morphology |
| MP:0010115 | abnormal embryonic cloaca morphology |
| MP:0011411 | abnormal gonadal ridge morphology |
| MP:0011257 | abnormal head fold morphology |
| MP:0011260 | abnormal head mesenchyme morphology |
| MP:0012187 | abnormal intraembryonic coelom morphology |
| MP:0005650 | abnormal limb bud morphology |
| MP:0006301 | abnormal mesenchyme morphology |
| MP:0008487 | abnormal mesonephros morphology |
| MP:0011256 | abnormal neural fold morphology |
| MP:0005657 | abnormal neural plate morphology |
| MP:0005650 | abnormal limb bud morphology |
| MP:0006301 | abnormal mesenchyme morphology |
| MP:0008487 | abnormal mesonephros morphology |
| MP:0011256 | abnormal neural fold morphology |
| MP:0008487 | abnormal mesonephros morphology |
| MP:0011256 | abnormal neural fold morphology |
| MP:0005657 | abnormal neural plate morphology |
| MP:0002151 | abnormal neural tube morphology/development |
| MP:0002825 | abnormal notochord morphology |
| MP:0002884 | abnormal pharyngeal arch morphology |
| MP:0013231 | abnormal pharyngeal groove morphology |
| MP:0013232 | abnormal pharyngeal membrane morphology |
| MP:0006031 | abnormal pharyngeal pouch morphology |
| MP:0012496 | abnormal pleuropericardial membrane morphology |
| MP:0002399 | abnormal pluripotent precursor cell morphology/development |
| MP:0013217 | abnormal posterior definitive endoderm morphology |
| MP:0003885 | abnormal rostral-caudal body axis extension |
| MP:0012253 | abnormal septum transversum morphology |
| MP:0001688 | abnormal somite development |
| MP:0002861 | abnormal tail bud morphology |
| MP:0011258 | abnormal tail fold morphology |
| MP:0001674 | abnormal triploblastic development |
| MP:0011835 | abnormal urogenital fold morphology |
| MP:0011853 | abnormal urorectal septum morphology |
| MP:0003988 | disorganized embryonic tissue |
| MP:0013241 | embryo tissue necrosis |
| MP:0008932 | abnormal embryonic tissue physiology |
| MP:0003890 | abnormal embryonic-extraembryonic boundary morphology |
| MP:0002086 | abnormal extraembryonic tissue morphology |
| MP:0001726 | abnormal allantois morphology |
| MP:0005029 | abnormal amnion morphology |
| MP:0011199 | abnormal amniotic cavity morphology |
| MP:0002836 | abnormal chorion morphology |
| MP:0011202 | abnormal ectoplacental cavity morphology |
| MP:0003396 | abnormal embryonic hematopoiesis |
| MP:0011200 | abnormal extraembryonic coelom morphology |
| MP:0010736 | abnormal extraembryonic ectoderm morphology |
| MP:0001724 | abnormal extraembryonic endoderm formation |
| MP:0006323 | abnormal extraembryonic mesoderm development |
| MP:0011203 | abnormal parietal yolk sac morphology |
| MP:0011711 | abnormal placenta morphology |
| MP:0011197 | abnormal proamniotic cavity morphology |
| MP:0001725 | abnormal umbilical cord morphology |
| MP:0011201 | abnormal visceral yolk sac cavity morphology |
| MP:0001718 | abnormal visceral yolk sac morphology |
| MP:0003229 | abnormal vitelline vasculature morphology |
| MP:0002582 | disorganized extraembryonic tissue |
| MP:0004264 | abnormal extraembryonic tissue physiology |
| MP:0004966 | abnormal inner cell mass proliferation |
| MP:0009781 | abnormal preimplantation embryo development |
| MP:0011186 | abnormal visceral endoderm morphology |
| MP:0012028 | abnormal visceral endoderm physiology |
| MP:0001730 | embryonic growth arrest |
| MP:0003984 | embryonic growth retardation |
| MP:0005379 | endocrine/exocrine gland phenotype |
| MP:0002163 | abnormal gland morphology |
| MP:0002164 | abnormal gland physiology |
| MP:0005378 | growth/size/body region phenotype |
| MP:0009701 | abnormal birth body size |
| MP:0005451 | abnormal body composition |
| MP:0000385 | abnormal body wall morphology |
| MP:0004134 | abnormal chest morphology |
| MP:0000432 | abnormal head morphology |
| MP:0012719 | abnormal neck morphology |
| MP:0002089 | abnormal postnatal growth/weight/body size |
| MP:0004196 | abnormal prenatal growth/weight/body size |
| MP:0001270 | distended abdomen |
| MP:0004133 | heterotaxia |
| MP:0013328 | visceromegaly |
| MP:0005377 | hearing/vestibular/ear phenotype |
| MP:0002102 | abnormal ear morphology |
| MP:0003938 | abnormal ear development |
| MP:0000062 | abnormal inner ear morphology |
| MP:0003938 | abnormal middle ear morphology |
| MP:0002177 | abnormal outer ear morphology |
| MP:0003878 | abnormal ear physiology |
| MP:0005397 | hematopoietic system phenotype |
| MP:0002396 | abnormal hematopoietic system morphology/development |
| MP:0002429 | abnormal blood cell morphology/development |
| MP:0002398 | abnormal bone marrow cell morphology/development |
| MP:0004808 | abnormal hematopoietic stem cell morphology |
| MP:0000689 | abnormal spleen morphology |
| MP:0000703 | abnormal thymus morphology |
| MP:0001545 | abnormal hematopoietic system physiology |
| MP:0002396 | abnormal hematopoietic/development |
| MP:0002429 | abnormal blood cell morphology/development |
| MP:0002398 | abnormal bone marrow cell morphology/development |
| MP:0004808 | abnormal hematopoietic stem cell morphology |
| MP:0000689 | abnormal spleen morphology |
| MP:0000703 | abnormal thymus morphology |
| MP:0001545 | abnormal hematopoietic system physiology |
| MP:0002396 | abnormal hematopoietic/development |
| MP:0002429 | abnormal blood cell morphology/development |
| MP:0002398 | abnormal bone marrow cell morphology/development |
| MP:0004808 | abnormal hematopoietic stem cell morphology |
| MP:0000689 | abnormal spleen morphology |
| MP:0000703 | abnormal thymus morphology |
| MP:0001545 | abnormal hematopoietic system physiology |
| MP:0002396 | abnormal hematopoietic/development |
| MP:0002429 | abnormal blood cell morphology/development |
| MP:0002398 | abnormal bone marrow cell morphology/development |
| MP:0004808 | abnormal hematopoietic stem cell morphology |
| MP:0000689 | abnormal spleen morphology |
| MP:0000703 | abnormal thymus morphology |
| MP:0001545 | abnormal hematopoietic system physiology |
Figure 9. Examples of new MP phenotypes. A–C. “Thin motoric part of trigeminal nerve”. Original HREM sections through the head of a Polb° embryo (A, B) and a control (C). Box in A indicates section displayed in B. D “Blood in lymph vessels”, as appearing in an original HREM section through the neck of a 1700067K01Rik° embryo. Note the blood filled left lymph sac (asterisk). Use the right sided lymph sac (rls) as a control. E. Double lumen aortic arch. Surface model of the great intrathoracic arteries on top of an original HREM section of a Pdzk1° embryo. (Compare with 17). F. “Intramural bleeding in blood vessel wall” (arrowhead) in the descending aorta (da) of an Akap9° embryo from the DMDD pilot study. G–H. “Abnormal elbow joint morphology” Sagittal sections. Normal situation in a control (G). Fusion of humerus (h) und ulna material (u) in an Atp11a° embryo. aa, aortic arch; ah, adenohypophysis; bt, brachiocephalic trunk; da, descending aorta; dlaa, double lumen aortic arch; e, esophagus; h, humerus; l, lung; la, left atrium; lcc, left common carotid artery; le, lens; lsa, left subclavian artery; lx, larynx; mp, motoric part of trigeminal nerve; nc, nasal cavity; oc, optic cup; r, radius; ra, right atrium; rcc, right common carotid artery; rv, right ventricle; sc, spinal chord; tg, trigeminal ganglion; u, ulna; v, vertrebral body; III, 3rd ventricle; Scale bars: 1 mm.

Two other factors in our study might affect interpretation of the mutant phenotype data. 11 of the 42 lines examined in our study were judged subviable at weaning, rather than lethal. This number is too small to support meaningful comparison of the phenotypic spectrum between subviables and lethals. It is tempting to speculate that a difference in phenotype penetrance might underlie the difference in viability between the two groups, but there is no evidence to support this from the DMDD study so far (see Supplementary Figure 4). Even if a difference in penetrance was detected between lethal and subviable lines, interpreting its significance is far from simple as it raises an important and unresolved question: which phenotypes are responsible for embryo death? Many profound abnormalities that we detect may be compatible with life; equally, lethality may result from subtle structural changes. Without knowing which of the scored phenotypes are likely to cause lethality, it will be difficult, if not impossible, to establish if differences in their penetrance distinguish subviable from lethal lines. Add to this the additional difficulty that dams have a propensity to eat newborns that are not thriving well and there is a further complication in interpreting the data.
Table 7. New MP terms derived from embryo phenotyping. A list of the Mammalian Phenotype Ontology IDs along with their corresponding term name. These have been added to the ontology to allow annotation of abnormalities observed in the embryos which could not be adequately described by existing terms.

| MP:0013809 | absent pectinate muscle |
| MP:0013810 | absent brachiocephalic trunk |
| MP:0013812 | enlarged orbital veins |
| MP:0013813 | dilated hepatic portal vein |
| MP:0013814 | abnormal hepatic portal vein connection |
| MP:0013816 | absent digastric muscle |
| MP:0013817 | absent nasal cavity |
| MP:0013818 | abnormal oral cavity morphology |
| MP:0013819 | abnormal acromioclavicular joint morphology |
| MP:0013820 | absent optic cup |
| MP:0013823 | absent segment of anterior cerebral artery |
| MP:0013825 | small hypoglossal canal |
| MP:0013826 | absent hypoglossal canal |
| MP:0013827 | thin oculomotor nerve |
| MP:0013828 | thin facial nerve |
| MP:0013829 | thin splanchnic nerve |
| MP:0013830 | abnormal intrathoracic topology of vagus nerve |
| MP:0013831 | vagus nerve compression |
| MP:0013832 | thin vagus nerve |
| MP:0013833 | absent olfactory nerve |
| MP:0013834 | thin hypoglossal nerve |
| MP:0013835 | absent hypoglossal nerve |
| MP:0013836 | abnormal hypoglossal nerve topology |
| MP:0013837 | abnormal vagus nerve topology |
| MP:0013838 | small caudate nucleus |
| MP:0013840 | absent segment of posterior cerebral artery |
| MP:0013841 | abnormal lymphatic vessel topology |
| MP:0013842 | ductus venosus topology |
| MP:0013843 | hepatic portal vein stenosis |
| MP:0013844 | abnormal perichondrial ossification |
| MP:0013845 | abnormal eye muscle topology |
| MP:0013846 | retropharyngeal edema |
| MP:0013847 | retropleural edema |
| MP:0013848 | subcutaneous edema |
| MP:0013849 | absent abducens nerve |
| MP:0013850 | absent posterior commissure |
| MP:0013851 | abnormal Wolffian duct topology |
| MP:0013852 | abnormal Mullerian duct topology |
| MP:0013853 | abnormal hepatic portal vein formation |
| MP:0013855 | absent celiac artery |
| MP:0013857 | abnormal abdominal muscle morphology |
| MP:0013858 | abnormal azygos vein topology |
| MP:0013859 | abnormal vitelline vein connection |
| MP:0013860 | anastomosis between common carotid and vertebral artery |
| MP:0013861 | abnormal pancreas topology |
| MP:0013862 | abnormal cecum position |
| MP:0013864 | enlarged paraumbilical vein |
| MP:0013865 | abnormal dorsal pancreas topology |
| MP:0013868 | abnormal ventral pancreas topology |
| MP:0013869 | vascular diverticulum |
| MP:0013870 | absent proximal internal carotid artery segment |
| MP:0013871 | abnormal stapedial artery topology |
| MP:0013873 | abnormal ductus venosus morphology |
| MP:0013874 | abnormal ductus venosus topology |
| MP:0013875 | trigeminal neurona |
| MP:0013876 | absent ductus venosus valve |
| MP:0013877 | abnormal ductus venosus valve morphology |
| MP:0013878 | abnormal ductus venosus valve topology |
| MP:0013879 | duplication of ductus venosus |
| MP:0013880 | absent ductus venosus |
| MP:0013913 | absent rib-vertebral column attachment |
| MP:0013914 | absent intracranial segment of vertebral artery |
| MP:0013915 | abnormal brachial plexus morphology |
| MP:0013916 | decreased intestine length |
| MP:0013917 | persistent right 6th pharyngeal arch artery |
| MP:0013918 | abnormal endolymphatic sac topology |
| MP:0013923 | small prevertebral sympathetic ganglia |
| MP:0013924 | abnormal dural venous sinus morphology |
| MP:0013925 | abnormal vascular plexus formation |
| MP:0013926 | absent neurohypophysis |
| MP:0013927 | abnormal facial nerve topology |
| MP:0013928 | thin motoric part of trigeminal nerve |
| MP:0013929 | absent eye muscles |
| MP:0013930 | abnormal digastic muscle connection |
| MP:0013931 | abnormal olfactory bulb position |
| MP:0013932 | fragmented Meckel's cartilage |
| MP:0013933 | short Meckel's cartilage |
| MP:0013934 | supratentorial ventricles enlargement |
| MP:0013935 | basal brain tissue herniation |
| MP:0013936 | abnormal thymus topology |
| MP:0013937 | absent lobe of thyroid gland |
| MP:0013938 | abnormal esophagus topology |
| MP:0013943 | abnormal ureter topology |
The lines we have studied fall roughly equally between those containing an insertion into the targeted gene (tm1a alleles) and those in which recombination has removed both a gene exon and the neomycin selection cassette (tm1b alleles). Interestingly, our data clearly reveals that tm1b alleles show greater penetrance of phenotypes than those containing the tm1a insertion. This may reflect the potential of tm1a alleles to be hypomorphic, and might also be influenced by their retention of the neo selection cassette.

It is also worth noting the several practical lessons which have become evident through the course of DMDD studies and which may be of value for similar embryo phenotyping programmes.

The most pressing of these is basing phenotype detection on comparison of each mutant embryo with an appropriately staged normal counterpart14. Embryos harvested at E14.5 vary markedly in their developmental progress and many tissues and organs are actively remodelled during this period. This is most obvious for the topology of the intestine, the position of the palatal shelves and the interventricular communication between left and right sides of the heart. Only with precise developmental staging is accurate phenotyping of these features possible8.

Whilst the precise range and detail of phenotypes that can be scored will necessarily be dictated by the nature of the imaging

| MP:0013944 | persistent cloacal membrane |
| MP:0013945 | abnormal elbow joint morphology |
| MP:0013946 | abnormal perirectal tissue morphology |
| MP:0013947 | abnormal paraaortic body morphology |
| MP:0013948 | intraembryonal intestine elongation |
| MP:0013949 | fusion of axis and occipital bones |
| MP:0013950 | abnormal dorsal root ganglion topology |
| MP:0013951 | abnormal descending aorta topology |
| MP:0013952 | retro-esophageal left subclavian artery |
| MP:0013953 | left sided brachiocephalic trunk |
| MP:0013963 | jugular vein stenosis |
| MP:0013964 | absent tongue muscles |
| MP:0013965 | abnormally deep median sulcus of tongue |
| MP:0013967 | abnormal infrahyoid muscle connection |
| MP:0013968 | multiple persisting cranialpharyngeal ducts |
| MP:0013969 | reduced sympathetic cervical ganglion size |
| MP:0013970 | absent connection between subcutaneous lymph vessels and lymph sac |
| MP:0013971 | blood in lymph vessels |
| MP:0013972 | occipital vertebra |
| MP:0013973 | abnormal hepatic vein connection |
| MP:0013974 | abnormal coronary vein connection |
| MP:0013975 | abnormal coronary sinus connection |
| MP:0013976 | abnormal left vena cava superior connection |
| MP:0013977 | symmetric azygos veins |
| MP:0013978 | abnormal carotid artery origin |
| MP:0013979 | abnormal subclavian artery origin |
| MP:0013980 | abnormal pulmonary artery origin |
| MP:0013981 | double lumen aortic arch |
| MP:0013982 | inverse situs of great intrathoracic arteries |
| MP:0013984 | abnormal superior mesenterial vein connection |
| MP:0013985 | abnormal umbilical vein topology |
| MP:0013986 | abnormal vitelline vein topology |
| MP:0013987 | absent intrahepatic inferior vena cava segment |
| MP:0013988 | absent portal vein segment |
| MP:0013989 | symmetric hepatic veins |
| MP:0013991 | abnormal common iliac artery origin |
| MP:0013992 | persistent dorsal ophthalmic artery |
| MP:0013993 | anastomosis between basilar artery and common carotid artery |
| MP:0013994 | abnormal parasellar internal carotid artery branch morphology |
| MP:0013995 | abnormal external carotid artery origin |
| MP:0013996 | abnormal vertebral artery origin |
| MP:0013997 | abnormal internal carotid artery topology |
| MP:0013998 | absent canalicular internal carotid artery segment |
| MP:0013999 | abnormal parasellar internal carotid artery |
| MP:0014000 | anastomosis between internal carotid artery and basilar artery |
| MP:0014001 | abnormal vertebral artery topology |
| MP:0014002 | absent extracranial vertebral artery segment |
| MP:0014003 | additional anastomosis between intracranial vertebral arteries |
| MP:0014004 | absent basilar artery segment |
| MP:0014006 | absent posterior communicating artery |
| MP:0014008 | absent labyrinthine artery |
| MP:0014009 | anastomosis between middle cerebral arteries |
| MP:0014011 | abnormal ovary tissue architecture |
| MP:0014017 | abnormal Wolffian duct connection |
| MP:0014018 | embryo tumor |
| MP:0014019 | embryo cyst |
| MP:0014020 | intramural bleeding in blood vessel wall |
| MP:0014021 | heterochrony |
| MP:0014022 | abnormal duodenum topology |
modality and the method of phenotype identification (compare, for example 15,16, with the manual annotation used in the present study), a common challenge is the development of protocols to minimise occurrence or subsequent scoring of apparent abnormalities that are more likely artefacts of sample preparation or processing. These can range from the more obvious ruptures of the embryo skin or damaged external features during dissection, to tissue shrinkage or swelling (causing organ deformation) as a result of dehydration, fixation or embedding. Finally, the power of phenotypic screens such as DMDD to inform our understanding of developmental disease rests heavily on the detail with which abnormalities are scored. However, the very complexity we have seen this generates makes it all the more urgent to distinguish phenotypes not just through the nature of the morphological abnormality, but through its capacity, individually or in concert with others, to compromise subsequent fetal survival.

Data availability
Dataset 1 Zenodo: 10.5281/zenodo.16350618

Dataset 2 Zenodo: 10.5281/zenodo.26889919

The cumulative list of all scored phenotypes analysed in this study is presented in Dataset 1 (homozygous mutants) and Dataset 2 (wild type embryos). The intermediate and high level slims of the MP ontology used in the analysis are presented in Supplementary table 2 and Supplementary Table 3. All data used in this study is also available from the DMDD web site (https://dmdd.org.uk) where phenotype annotations are available in tabular format by embryo and by line. In addition, they are identified at their appropriate locations within each 3D dataset of embryo images, which can be viewed in all three orthogonal section planes.

Author contributions
RR, JW, CT, CM, ET and AG identified lethal lines and provided embryos; EH, LF, AM, FP and TM carried out HREM imaging, SG, LR, JR, DS and WW identified phenotypes; RW performed data analysis; CMcG and RW designed and maintained the DMDD web portal; JS, ER and DA contributed to the design of the study; RW, SG, WW and TM prepared the manuscript. All authors were involved in the revision of the draft manuscript and have agreed to the final content.

Competing interests
No competing interests were disclosed.

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgements
We are grateful for the contributions made by past and present members of the DMDD consortium and the support of their institutions, without which the DMDD programme would not be possible.

Supplementary material
Supplementary Figure 1: Embryo Homozygous mutant embryo numbers analysed for each mutant line.
The number of annotated embryos scored for each of the 42 lines was used to plot the variation in numbers of embryos analysed per line.
Click here to access the data.

Supplementary Figure 2: Distribution of homozygous mutant embryo phenotypes amongst DMDD mutant lines (high level MP ontology slim).
Data from the global analysis of the frequency of phenotype terms (see Materials and Methods) is plotted to show the penetrance of phenotypes scored for each line, indicated by a colour gradient from light yellow (no penetrance) to dark red (100% penetrance). Each line is labelled after the symbol of the gene disrupted (see also Table 1), and the number of homozygous mutant embryos analysed for each line is shown. Phenotype annotations are summarised using the high level DMDD ontology slim.
Click here to access the data.

Supplementary Figure 3: Distribution of homozygous mutant embryo phenotypes amongst DMDD mutant lines (intermediate level MP ontology slim).
As in Supplementary Figure 2, except that phenotype annotations are summarised using the intermediate level DMDD ontology slim.
Click here to access the data.
Supplementary Figure 4: Penetrance of mutant embryo phenotypes in lethal and subviable mutant lines.

Data from the global analysis of the frequency of phenotype terms (see Materials and Methods) was plotted using embryos of the same genetic background (C57BL/6N;C57BL/6NTac) to show the number of lines falling into each of the observed phenotype categories (using the MP ontology intermediate slim). Each of the distinct penetrance categories is colour coded and data is ordered according to line frequency. (A) shows the penetrance distribution in the combination of lethal and subviable lines; (B) shows the equivalent plot for lethal lines alone.

Supplementary Table 1A and 1B: Embryo phenotypes, organised by frequency.

The data from the global analysis of the frequency of phenotype terms (see Materials and methods) is presented in a structured fashion showing the relationship between Mammalian Phenotype Ontology terms included in the DMDD high level ontology slim, the DMDD intermediate level ontology slim, and the original annotation terms. The first three columns list the ID, term and frequency of DMDD high level ontology terms, columns 4–6 list the ID, term and frequency of the intermediate level ontology terms that cluster under the high level term listed in column 1, and for each intermediate level term the annotation phenotype terms are shown in order of frequency in columns 7–9. Column 10 lists the lines in which the phenotype listed in columns 7 was observed. The table rows are ordered according to the frequency of the high level ontology terms, intermediate ontology terms and original annotation terms. **Table 1A**: homozygous mutant embryos; **Table 1B**: wildtype embryos.

Table 1A:

Table 1B:

Supplementary Table 2: High level MP ontology slim used by DMDD.

A list of the Mammalian Phenotype Ontology IDs and names of terms selected as the high level ontology slim.

Supplementary Table 3: Intermediate level MP ontology slim used by DMDD.

A list of the Mammalian Phenotype Ontology IDs and names of terms selected as the intermediate level ontology slim.

Supplementary Tables 4 and 5: All embryo phenotypes from lethal and sub-viable lines scored by DMDD to date.

The tables list the annotation data that is the basis of the study. For every annotation the gene symbol, MGI_ID, allele symbol, DMDD_ID, MP term, ID and name is listed. In some cases the same MP term is listed more than once for a specific embryo (DMDD_ID), indicating the phenotypic abnormality was observed more than once in that embryo.

Supplementary Table 4: homozygous mutant embryos

Supplementary Table 5: wild type embryos
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Version 2

Reviewer Report 19 June 2017

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Lydia Teboul
The Mary Lyon Centre, Medical Research Council Harwell Institute, Didcot, UK

The authors have addressed all of my concerns. I thank the authors for the additional data and analysis which now provide a much more complete picture.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 21 March 2017

https://doi.org/10.21956/wellcomeopenres.11724.r21156

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Nadia Rosenthal
National Heart and Lung Institute, Imperial College London, London, UK

The authors have addressed all of my concerns and the addition of the very interesting WT data has improved the manuscript. I would only add that there is one additional, and perhaps more likely, explanation for the identification of phenotypes in the WT dataset. Because the controls were littermates from mutant generation, the “mutagenic load” referenced in the discussion could be derived from the targeted ES cells, rather than the C57BL/6N background line itself. However, the manuscript is perfectly acceptable as it stands, and the authors can include this possibility at their discretion.
**Competing Interests:** No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

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**Lydia Teboul**

1. The Mary Lyon Centre, Medical Research Council Harwell Institute, Didcot, UK
2. The Mary Lyon Centre, Medical Research Council Harwell Institute, Didcot, UK

The Deciphering Mechanisms of Developmental Disorders consortium presents a systematic study of the morphology of mutant embryos from 42 lines developed in the frame of the International Phenotyping Consortium. The lines chosen for this study were selected as they are homozygous lethal or subviable at weaning but viable at E14.5.

The authors employ High Resolution Episcopic Microscopy to capture 3D images of the embryos, providing exquisitely detailed documentation of embryo morphologies. They exploit this rich dataset with a systematic and in depth annotation of morphological defects which they record using appropriate levels of MP terms.

The result is a survey of impressive scope in terms of annotation depth and volume of data, and a superb effort of data organisation and analysis so the great complexity of the dataset can be distilled to overall observations and discussion points. This organisation effort yielded a really useful framework for systematic analysis of the morphology of mouse mutant of that stage.

The authors conclude that a salient point of the work is the great variability of penetrance of the morphological phenotypes they find among these mutant embryos of the same isogenic genetic background.

Although the variable expressivity of phenotype between different individuals of a same mutant line isn't a new concept, the unexpected result of the study is the extend to which phenotypes (even when grouped in broad categories such as “organ affected”) vary in penetrance, albeit that these mutants share the broadest of phenotype which is lethality.

However, the authors restrict their analysis to the variability amongst mutants and they mention in the discussion an on-going systematic analysis of WT embryos, which will provide key information to put in context the observations collated in this article.
Whereas the article is an excellent effort of presenting a complex dataset with clarity and granularity and documenting variability of morphology amongst samples, the data presented do not allow the reader to identify the reason(s) of this variability in the absence of key information. Three major points should be addressed:

- The authors made the unusual choice of not presenting baseline data on the morphology of wild-type mutants (littermates) produced in the study. Such data, surveying significant groups of control embryos, would be essential to establish the link between mutations and described phenotypes. In the absence of this data, any reference to a causal link between phenotypes and mutation should be removed from the article.

- Both targeted traps (tm1a) and null (tm1b and CRISPR induced deletions) alleles are employed in the study. Both the presence of a selection cassette and the unpredictability of efficiency of trapping cassette(s) could form the basis of at least some of the variability shown in this study. An evaluation of variability (particularly using slim terms) within each of these 2 groups of alleles would help to address this point.

- Subviable lines show by definition a partially penetrant phenotype and contribute to a quarter of the mutant studied. An evaluation of variability (particularly using slim terms) within lethal and subviable as separate alleles groups would discriminate whether variability of morphology is particularly occurring among subviable lines.

Minor points:

- Methods should detail information that permit the appraisal of materials used in the study, detailing the genetic background of stem cells and animals employed for germline transmission, and further breeding, including whether homozygotes were used to produce embryos to analyse subviable lines.

- Methods should outline the steps taken to limit manual annotation variability (i.e. secondary calling or benchmarking between annotators).

- All titles and text should precisely detail when lethal or both lethal and subviable mutations are presented.

**Competing Interests:** No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 06 Mar 2017

Tim Mohun

The authors made the unusual choice of not presenting baseline data on the morphology of wild-type mutants (littermates) produced in the study. Such data, surveying significant groups of control embryos, would be essential to establish the link between mutations and described phenotypes. In the absence of this data, any
reference to a causal link between phenotypes and mutation should be removed from the article.

We have included the wild type phenotype data in the revised version of the manuscript (see the detailed response to Rosenthal/Murray for more details).

Both targeted traps (tm1a) and null (tm1b and CRISPR induced deletions) alleles are employed in the study. Both the presence of a selection cassette and the unpredictability of efficiency of trapping cassette(s) could form the basis of at least some of the variability shown in this study. An evaluation of variability (particularly using slim terms) within each of these 2 groups of alleles would help to address this point.

The revised manuscript now includes separate analysis of phenotypes for the 22 tm1a alleles compared with 20 complete nulls (19 tm1b and 1 CRISPR). With either allele, blood vessel, heart and brain morphology remain amongst the most commonly observed abnormalities. However, with such relatively small numbers, we feel there is little more that can usefully be concluded from comparison of individual phenotype prevalence, since this will be heavily influenced by the distinct gene identities within each allele group. In contrast, there is a clear difference in phenotype penetrance between the two groups: phenotypes are clearly more penetrant from tm1b alleles (see new Figure 8A and 8B). We presume that this reflects the fact that whilst mutations based on tm1a alleles have the potential to be hypomorphic, those converted from tm1a to tm1b contain an exon deletion (and no longer carry the neo selection cassette).

Subviable lines show by definition a partially penetrant phenotype and contribute to a quarter of the mutant studied. An evaluation of variability (particularly using slim terms) within lethal and subviable as separate alleles groups would discriminate whether variability of morphology is particularly occurring among subviable lines.

We presume that the reviewer is wondering whether the difference between lethal and subviable lines is a result of differing degrees of penetrance of phenotypes that result in embryo death. Answering this point is not as simple as it might appear as it touches on a much more profound issue raised by studies such as ours. Whilst we are able to distinguish a remarkable number of different structural abnormalities by virtue of the resolution HREM imaging affords, it may not be at all clear which of these results in embryo lethality. Many profound abnormalities may be compatible with life and lethality may also result from structurally subtle changes. Without knowing which of the scored phenotypes are likely to cause lethality, it will be difficult if not impossible to establish of differences in their penetrance distinguish subviable from lethal lines. Add to this the additional difficulty that dams have a propensity to eat newborns that are not thriving well and there is a further complication in interpreting the data.

We have nevertheless reexamined the phenotype data in order to compare the results separately for lethal and subviable lines (new Supplementary Figure 4). From this it is clear
that there is insufficient data from subviable lines to draw unequivocal conclusions. Overall, the approximate prevalence of particular phenotype terms (using the intermediate slim) appears broadly similar to that of lethals, but for most of these, the numbers of affected lines are too few to make useful estimates of penetrance.

**Minor points:**

1. Full details of genetic background and mutant allele are now provided for each line (revised Table 1).

2. All phenotyping was performed according to a standardised and sequential procedure, as mentioned in Material and methods. The data from each embryo was independently reviewed by a second anatomist and any discrepancies resolved by joint agreement.

3. We have amended titles and text to ensure that the distinction between lethal and subviable lines is clear where necessary.

**Competing Interests:** No competing interests were disclosed.
data. In addition to minor issues detailed below, there are two major gaps, however, that must be addressed.

1. There is no description of the number of control embryos screened or the incidental rate of hits for each phenotype in the DMDD list. Given the focus of the paper on the variability of phenotype penetrance and the number of phenotypes with an “n=1”, it is impossible to draw conclusions without this information. While the authors allude to a manuscript in preparation, it is actually essential data for this paper.

2. Similarly, there is no description of how the authors account for global developmental delay in mutants, which can lead to many “phenotypes” that are merely the result of slowed/retarded development or variability in developmental timing between litters. For example, at E14.5, one would expect a high rate of cleft palate in mutants that have some level of overall delay, or in entire delayed litters, as the palate is elevating and fusing at that time point. This raises the following questions: are controls from each litter collected? How is uniform staging assured? Are “delayed” embryos compared to a stage-matched control? Again, the authors allude to another manuscript, but some of this information needs to be included here to assure the MP calls do not have trivial explanations.

Minor points:

1. While the brief description of the animal resource and use of website citation is acceptable, given the main finding of variable penetrance, the authors should make a point of describing the isogenic genetic background and the nature of the alleles (tm1a or tm1b) in the methods and results.

2. It’s not entirely clear if this was a set of 42 genes that were lethal/subviable at wean, or if this was a select set of lethal genes that were viable/subviable (present) at E14.5. Given the comments in the discussion about lines lethal at E9.5 or earlier, I assume the latter. This should be spelled out.

3. Mouse gene symbols should be italicized.

4. Apart from Table 1, the tables are too large and make reading a PDF a somewhat painful process. These might not be easily compressed, so most of the information should be moved to a supplemental file.

Competing Interests: No competing interests were disclosed.

We confirm that we have read this submission and believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however we have significant reservations, as outlined above.

Author Response 06 Mar 2017

Tim Mohun

There is no description of the number of control embryos screened or the incidental rate of hits for each phenotype in the DMDD list. Given the focus of the paper on the variability of phenotype penetrance and the number of phenotypes with an “n=1”, it is
impossible to draw conclusions without this information. While the authors allude to a manuscript in preparation, it is actually essential data for this paper.

The revised manuscript now includes the complete phenotype data obtained for 114 wild type embryos. This comprises 56 phenotype calls, affecting 32 embryos, originating from 28 lines (revised Tables 1, 2B and Supplementary Table 5). 21 of the 56 phenotype calls (38%) are accounted for by only 6 embryos, (indicating the skewing effect of a small number of abnormal embryos), most affected embryos showing only a single phenotype. This is in marked contrast to the finding of many different phenotypes in individual mutant embryos. The phenotypes of wild types vary in character, ranging from apparently minor differences (e.g. in blood vessel morphology) to a few major abnormalities (e.g. absent kidney). Each one is rare amongst the population of wild type embryos analysed and affects only a single wild type embryo within the line. Only 10 phenotypes (15 phenotype calls) overlap between mutant embryos and their wild type siblings and these affect only 10 of the 41 lines for which wild type embryos have been assessed (Table 3). As we discuss in the revised Results and Discussion sections, these data raise 3 related questions: Why are phenotypes detected in genetically wild type embryos? Are there “background” phenotypes associated with the C57BL/6N line that contribute to the mutant phenotypes scored? Is there any evidence for “background” phenotypes associated with an individual knockout line?

We think there are several possible explanations for finding phenotypes amongst wild type embryos. One possibility is that the mouse strain that has been used for engineering knockout lines carries a “background load” of abnormalities, previously unappreciated. Ours is the first systematic study on sufficiently large scale and employing sufficiently high resolution imaging to detect such abnormalities. Amongst the phenotypes identified, none shows significant prevalence that might be expected if it was a strain-specific abnormality. Another possible explanation is that abnormalities arise as a consequence of de novo mutation and the frequency we detect reflects the high sensitivity that results from HREM imaging. Lastly, at least with the less apparently severe abnormalities, it is possible that some of these in fact represent outliers on spectrum of normal morphological variation and should not be considered genuine abnormalities. This highlights an important issue confronting phenotyping studies: the dearth of large-scale and systematic studies examining normal embryo morphology that can set a reliable benchmark for distinguishing abnormalities from normal variation. In this light, phenotype data may need revision as cumulative experience improves our ability to distinguish abnormalities from variation amongst wild types.

Whatever the explanation, it is clear that neither the frequency, prevalence nor nature of the phenotypes identified in wild type embryos impact significantly on the assignation of phenotypes amongst the homozygous mutant embryos.

Similarly, there is no description of how the authors account for global developmental delay in mutants, which can lead to many “phenotypes” that are merely the result of slowed/retarded development or variability in developmental timing between litters. For example, at E14.5, one would expect a high rate of cleft palate in mutants that have some level of overall delay, or in entire delayed litters, as the palate is elevating
and fusing at that time point. This raises the following questions: are controls from each litter collected? How is uniform staging assured? Are “delayed” embryos compared to a stage-matched control? Again, the authors allude to another manuscript, but some of this information needs to be included here to assure the MP calls do not have trivial explanations.

We believe it is important to distinguish between the effect of precise developmental stage of phenotyping and the issue of developmental retardation or delay. We can now reference the published study we mentioned that addresses these very questions (Geyer et al. 2017, J. Anat. in press). We do indeed collect wild type controls from each litter but our experience has demonstrated that precise stage matching of mutants with controls is essential to underpin accurate phenotyping. To facilitate this, we have analysed a large number of wild type embryos from the same genetic background as the that used for engineering of mutant lines. We have developed a system that can reliably distinguish five sub-stages within the span of Theiler stages 21 to 22 that are collected during E14.5, enabling us to compare each mutant embryo against precise, developmental stage-matched controls. Careful study and comparison of these has identified those changes (such as fusion of palatal shelves) which occur during the window of development that we observe. By combining qualitative comparisons with quantitative morphometry and statistical analysis, we are able to distinguish what can be considered genuine abnormalities from features that show either rapid developmental change or significant variability in the developmental timing of their appearance.

A more precise staging system also allows us to phenotype homozygous mutant embryos accurately, even though they frequently show some developmental delay, since we are able to compare them to controls at the equivalent stage of development. It also allows us to score instances of heterochrony where this affects individual (or a limited subset of) organs or tissues. By analysing a large number of wild type embryos harvested at E14.5, we have identified the spread and distribution of individual developmental sub stages that might be expected, and on this basis have a robust, statistical definition for global developmental retardation. Our studies do not allow us to identify why such retardation is relatively common amongst mutant embryos, but do offer some interesting pointers that we have commented upon. Retardation is, for example, much more common in mutant embryos showing cardiovascular defects (Geyer et al. 2017, J. Anat. in press). Furthermore, a surprisingly large proportion of mutants show abnormalities in their placental structure, and this may perhaps impact on their overall growth and development (unpublished data).

**Minor points**

1. The genetic background and details of each allele are now included in revised Table 1. 39 of the 42 lines analysed are on an identical background (C57BL/6N;C57BL/6NTac). 22 lines contain the tm1a allele, 19 contain tm1b and 1 line was produced using CRISPR.

2. The “Embryos” section of Materials and Methods details how the 42 lines were designated as lethal or subviable at wean (P14).
3. Mouse gene symbols have been italicised.

4. It was not possible for the larger tables to be moved to supplementary files; this is an unfortunate limitation of the online presentation method. Wellcome Open Research requested that the tables were included as figures rather than supplementary data and we agree that it is helpful for the reader to see the nature of the data. We had hoped that the individual files could also be downloaded in their spreadsheet format to allow full interrogation but the interface does not currently allow this. We have requested this change but in the meantime large tables are now also included in supplemental spreadsheet files to allow the reader to search and filter the data as required.

**Competing Interests:** No competing interests were disclosed.

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David Brook

1 School of Life Sciences, University of Nottingham, Nottingham, UK
2 School of Life Sciences, University of Nottingham, Nottingham, UK

We live in interesting times. Election outcomes are unpredictable. People are unpredictable and now as Wilson et al. report even the consequences of specific mutations are significantly less predictable than we might expect.

The Deciphering the Mechanisms of Developmental Disorders (DMDD) programme aims to analyse 240 embryonic lethal mouse knockout lines over a five-year period to study genes essential for mouse embryonic development and survival. This paper provides the first report on results gathered thus far. Wilson et al performed a detailed assessment of morphological abnormalities at stage E14.5 in 220 embryos from 42 novel mouse gene knockout lines. High Resolution Episcopic Microscopy was used to detect abnormalities at a scale from whole organs and tissues down to individual nerves and blood vessels. They report multiple abnormalities in virtually all of the embryos studied. They generated a wealth of information; in excess of 1.6 million images including more than 700,000 transverse sections to detail the incidence of structural abnormalities in 209 of the 220 embryos analysed. Eleven of the embryos from nine different lines were apparently normal.

To provide systematic phenotypic data Mammalian Phenotype (MP) ontology terms were used to classify abnormalities with high and intermediate levels. This allowed the authors to calculate a penetrance score for the terms in each of the mutant lines and to assign these to a quartile percentage group. Only 3 phenotypes were 100% penetrant and over half of the abnormalities
had a penetrance score under 25%.

Approximately one third of mouse gene knockouts are lethal and 60% of lethal lines entering the DMDD programme fail to provide homozygous mutant offspring by E14.5 with half of those being lethal prior to E9.5. Thus, as the authors point out, the data presented are from a subset of lethal lines. However, the most striking aspect of this study is the variability in penetrance of virtually all of the phenotypes analysed.

Recent studies sequencing human exome DNA has identified a high frequency of loss of function mutations. A study by Lek et al 2016 examined more than 60,000 human exomes and reported predicted homozygous loss of function genotypes in 1775 genes. On average there are 35 homozygous gene deletions in each human. Thus the comment by Wilson et al in the present paper is particularly pertinent; relating these findings to human developmental disease will require further sophisticated analysis. It would appear that homozygous loss of function mutations are more common than previously realised and, furthermore, the consequences of loss of function mutations are much more variable than previously realised. It will not be trivial to unmask the causes of this variability. We are only just beginning to scratch the surface of understanding the consequences of loss of function mutations in both mice and humans.

I have only one minor suggestion. On p4 3 lines from the bottom, the sentence starting "The Brd2 and Tcf712 alleles showed a similar, but less pronounced, conservation of phenotype.." requires clarification. Do they mean similar to Atp11a, to each other, or to both?

References
1. Lek M, Karczewski KJ, Minikel EV, Samocha KE, et al.: Analysis of protein-coding genetic variation in 60,706 humans. Nature. 2016; 536 (7616): 285-91 PubMed Abstract | Publisher Full Text

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 06 Mar 2017
Tim Mohun

The sentence on p4 referenced in the reviewer’s comment describes a trend in the similarity of phenotypes across all of the embryos within a particular mutant line. So we are not comparing the phenotypes between lines, but whether there is consistency between different embryos within any individual line.

Competing Interests: No competing interests were disclosed.