Placentation defects are highly prevalent in embryonic lethal mouse mutants

Vicente Perez-Garcia1,2*, Elena Fineberg1,2*, Robert Wilson3, Alexander Murray1,2, Cecilia Icoresi Mazzeo4, Catherine Tudor4, Arnold Sienert1,2, Jacqueline K. White5, Elizabeth Tuck4, Edward J. Ryder4, Diane Gleeson4, Emma Siragher5, Hannah Wardle-Jones5, Nicole Staudt4, Neha Wall5, John Collins4, Stefan Geyer5, Elisabeth M. Busch-Nentwich6,7, Antonella Galli8, James C. Smith3, Elizabeth Robertson7, David J. Adams4, Wolfgang J. Weninger5, Timothy Mohun3 & Myriam Hemberger1,2

Systematic identification of the genes required for normal embryogene-sis is essential if we are to successfully unravel the molecular framework that underpins embryo development. Such knowledge will help to identify genetic causes of developmental abnormalities that manifest during pregnancy or at birth, and comprise a considerable health burden. Large-scale phenotyping efforts have consistently found that 25–30% of mouse gene knockouts result in non-viable offspring1–5. In almost all studies of developmentally critical genes, research has focused on the impact of the mutation on the embryo. By contrast, little attention has been paid to the possible effects of these mutations on extraembryonic tissues, almost certainly resulting in underrepresentation of placental phenotypes in public databases. The Mouse Genome Informatics (MGI) database, for example, shows extraembryonic defects in only 10% of embryonic-lethal strains. Gaining a more accurate view of the actual frequency of placental abnormalities is crucially important for our understanding of the contribution of this vital organ to the aetiology of developmental defects and congenital abnormalities6.

Several groundbreaking studies have highlighted the essential role of extraembryonic tissues for normal development and long-term health. Placental insufficiency results in intrauterine growth retardation and, as a consequence, can cause fetal programming effects that predispose to later-onset disease7,8. Moreover, tetraploid complementation9 or conditional gene ablation experiments have identified embryonic lethal phenotypes in which normal development can be entirely rescued solely by providing the embryo with a wild-type placenta10–15.

However, systematic efforts to discover the genes required for normal placental development are still missing. The Deciphering the Mechanisms of Developmental Disorders (DMDD; https://dmdd.org.uk) consortium16 is one of several ongoing programmes dedicated to identifying and characterizing embryonic lethal genes in the mouse. In addition to detailed phenotypic assessment of structural abnormalities in mutant embryos, the DMDD project also investigates the effect of each mutation on placental development. Here we report the analysis of placental morphology for 103 such lines. Our results reveal a markedly higher rate of placental phenotypes than had been previously appreciated, and a striking association of placental defects with specific abnormalities in the embryo itself. Our study identifies the placenta as a pivotal target organ for the effects of gene mutations that contribute to developmental demise.

Placental defects in embryonic–lethal mouse lines

We analysed 103 mouse knockout lines that fail to produce mutant offspring at the expected Mendelian frequency at postnatal day (P)14, but yielded mutant embryos at either embryonic day (E)14.5 or E9.5. Lines for which mutant conceptuses could not be recovered at E9.5 were not included in this screen. Of the 103 lines analysed, 82 were classified as P14 lethal, as no mutant offspring were recovered at that stage. The remaining 21 lines were termed sub-viable, with mutant pups constituting 13% or less of all offspring obtained, a proportion significantly below the 25% expected from heterozygous crosses (Fig. 1a, Supplementary Table 1). Similar criteria were applied to sub-categorise the P14 lethal group further according to viability at P14 (Fig. 1a).

Placentas of all lines were subjected to histopathological analysis at E9.5, E14.5 or both (Supplementary Table 1). As expected, less than 1% of wild-type placentas showed an abnormal phenotype. By contrast, in mutant placentas we detected dysmorphologies in 56 out of 82 (68%) of P14 lethal strains (Fig. 1b). Even when the P14 sub-viable lines were...
included, the placental phenotype rate was still 59%, a far higher frequency than the approximately 10% annotated in the MGI database (Fig. 1b). All genes associated with placental abnormalities in mutants were expressed in the trophoblast lineage of this organ (Extended Data Fig. 1a), lending support to the notion that they contribute directly to placental growth or function.

We also assessed the conceptuses for yolk sac defects, an extra-embryonic structure that is especially important for nutrient provision during the first half of gestation, before formation of the functional placenta at E9.5 (Supplementary Table 1). Because the yolk sac was routinely used for genotyping, it proved only possible to analyse this tissue in 66 lines. Among these, an abnormal yolk sac morphology was detected in 11% (7 out of 66) of cases, compared to approximately 6% annotated among prenatal lethals in the MGI database. Notably, all seven affected lines fell within the E9.5–E14.5 lethal group (7 out of 24 = 29%; Fig. 1c). Thus, although yolk sac defects that affect its structure or haematopoietic function may contribute to the lethality of some of these early lethal strains, they occur at a much lower frequency than placental abnormalities.

When scoring the occurrence of placental defects as a function of developmental stage, we found that almost every line that died before E14.5 exhibited placental abnormalities (40 out of 41; Fig. 1d, Supplementary Table 1). These findings demonstrate that mutations resulting in embryonic lethality between E9.5 and E14.5 are almost certainly associated with a defective placenta.

In line with the placenta being the essential nutrient-supplying organ from mid-gestation onwards, we also found that mutant E14.5 embryos in strains exhibiting a placental phenotype were shifted to a younger developmental stage compared to those in which placental development was normal (Fig. 1e, Extended Data Fig. 1c).

Categories of placental defects

To categorise the different types of defect, we examined the three main layers of the mature placenta: the labyrinth, which constitutes the main nutrient and gas-exchange surface; the junctional zone, which consists of spongiotrophoblast, glycogen cells and different giant cell subtypes; and the maternally derived decidua (Fig. 2, Extended Data Fig. 2a).

Haematoxylin and eosin histology (Extended Data Fig. 2b, c) was complemented with three histological staining methods to accurately classify the cellular and tissue composition defects in abnormal placen- tasa using a series of phenotype criteria (Fig. 2a, b). At E9.5, a frequently detected malformation affected the invagination of allantoic blood vessels into the chorionic ectoderm, a process crucial for the development of the labyrinth that will almost certainly result in developmental arrest (Fig. 2a, Extended Data Fig. 2b). At E14.5, by far the most prevalent abnormalities were defects in the growth and intricate organization of the fetal and maternal blood conduits within the labyrinth layer (Fig. 2b, d, Extended Data Fig. 2c). Because these abnormalities diminish the surface area available for nutrient transport, they will compromise fetal growth and survival.

Collectively, these histological characterizations of more than 300 mutant placenatas provide a vast resource for the research community (all data are available at https://dmdd.org.uk).

Critical nodes in placental development

We next examined whether the identity of genes associated with placental defects suggested specific molecular pathways that may be pivotal for the formation or function of this organ. For genes that affect placental morphology at E9.5 in mutants, this network analysis highlighted several functional gene clusters that centred around Lmnb12, Bap1 and Arhgef7 (Fig. 2e, Extended Data Fig. 1d). Similarly, several factors identified in the E14.5 analysis formed specific molecular nodes, for example, around Traf2, Nek9 and Rpgrip1l (Extended Data Fig. 1d). Although relatively few genes have been analysed for defects in extraembryonic tissues in the literature, it is obvious that a large fraction of network components identified in our analyses have been associated with embryonic phenotypes. It therefore seems highly likely that mutants for many of these functionally connected genes will also exhibit placental abnormalities.

Embryo and placenta defects are linked

Because the DMDD program scores both embryonic and placental defects, it provides a unique opportunity to assess co-associations between specific phenotypes. Importantly, DMDD phenotype calls are based on precise embryo sub-staging, and the analysis therefore excludes any apparent phenotypes that simply reflect the developmental delay prevalent among embryos with placental defects. Nevertheless, mutant mouse lines that exhibit placental abnormalities were enriched for specific E14.5 embryo phenotypes that were distinct from those with normal placenatas (Extended Data Fig. 3a).

Embryo phenotype categories that show significant statistical correlation with placental defects included abnormalities in the heart, brain and vascular system (Fig. 3a, Extended Data Figs 3b, 4a, b and Supplementary Table 2). In particular, this affected anomalies in forebrain development, heart chamber and septum morphology, subcutaneous oedema, and overall artery or vein topology (Fig. 3b–d, Extended Data Fig. 4c, d). These phenotype co-associations suggest co-regulatory or inter-dependent mechanisms during the development of particular
organ systems, notably between the placenta and morphogenesis of the brain, heart and vascular system.

**Trophoblast-specific gene functions**

Because the placenta comprises cell types of distinct lineage origins, a placental phenotype may be caused by trophoblast-intrinsic and/or extraembryonic mesoderm-derived endothelial cell defects. To determine trophoblast-specific functions of genes identified as important for placental development, we used CRISPR–Cas9-mediated ablation in trophoblast stem cells (TSCs)20–22 (Extended Data Fig. 5). We chose three genes for this analysis that caused lethality around E9.5–E10.5 when ablated: the tumour suppressor Bap1, the cell polarity complex member Cdx2 and the nucleotide binding protein-like factor Nubpl (Extended Data Fig. 6).

*Nubpl*-mutant TSCs exhibited a decreased stem-cell potential, as evidenced by lower expression levels of *Cdx2*, *Esrrb* and *Etf5*, which may explain the marked reduction in the size of the trophoblast compartment in *Nubpl*−/− placenta. Moreover, severely impaired upregulation of *Gcm1* (an early marker of syncytializing trophoblast) and lower expression levels of *Syna* and, to a lesser extent, *Synb* showed that differentiation towards the syncytiotrophoblast lineage was inhibited in the absence of *Nubpl* (Fig. 4a, Extended Data Fig. 7a). We also detected a prominent phenotype in *Bap1*-deficient TSCs, as they displayed increased expression of the key stem-cell markers *Cdx2* and *Esrrb* when grown under self-renewal conditions. When triggered to differentiate, *Bap1*−/− TSCs failed to upregulate markers of syncytiotrophoblast, sinusoidal trophoblast giant cells and glycogen cells (Fig. 4b, Extended Data Fig. 7b). These TSC differentiation defects may well contribute to the labyrinth formation phenotype evident in both *Nubpl* and *Bap1* mutants. By contrast, *Cdx2*-null TSCs were indistinguishable from wild-type (empty vector) controls (Extended Data Fig. 7c).

**Lineage origins of placental defects**

To gain further insights into trophoblast-intrinsic versus embryonic lineage-induced effects, we chose the same three genes that we studied in TSCs for conditional gene ablation in vivo. Thus, we used the *Sox2*-cre transgene to delete the function of these genes in the embryo, while leaving expression intact in the trophoblast-derived cells of the placenta and the visceral yolk sac endoderm23 (Fig. 5a).

---

Figure 2 | Summary of common placental defects and functional networks. a, Common phenotype criteria used to assess E9.5 mutant placentas (red denotes abnormality detected). Morph., morphology; Subv., sub-viable; TGC, trophoblast giant cell. 1110037F02Rik is also known as Virma; Fam21 is also known as Wathc2. b, E14.5 placental phenotypes in mutant strains. SpT, spongiotrophoblast; lab., labyrinth. c, Top, schematic representation of main structures of an E9.5 placenta. Bottom, in situ hybridization for spongiotrophoblast marker *Tphpa* and immunostaining against E-cadherin (Cdh1) on wild-type and mutant placentas, as indicated. Large red arrows highlight *Tphpa*-positive cells. Small red arrows in the Cdh1-stained wild-type placenta highlight nucleated blood cells in fetal blood vessels. Arrowheads in the Pigi−/− placenta demarcate sites of chorionic ectoderm invagination but absence of blood vessels. d, Top, schematic representation of the main structures of an E14.5 placenta. Bottom, examples of histological analyses of E14.5 wild-type and mutant placentas, as indicated. Red vertical lines in the *Tphpa* in situ hybridization images show thickness of the functional zone. The BSI-B4 isolecitin stains demarcate the three main placental layers, and the red rectangle highlights the severely reduced complexity of labyrinth vascularisation in the Traf2 mutant. Red arrowheads in the Cdh1 immunohistochemistry images point to widened blood spaces of the Cdh1-labelled syncytiotrophoblast; arrows denote fibrotic areas. Images e and d are representative of at least three independent mutants per line, see Methods. e, Network created using esyN (http://www.esyn.org) of known interactors of L3mbtl2.
Nubpl-null embryos associated with a heterozygous placenta were considerably more advanced in development than their complete knockout counterparts at E9.5 and could still be recovered up to E11.5, a stage when the complete knockout was already resorbed (Fig. 5a, Extended Data Fig. 8a). Histological examination of the E9.5 and E11.5 placentas showed that the trophoblast expansion, syncytiotrophoblast differentiation and labyrinth vascularization defects were seemingly fully rescued in the conditional knockouts (Fig. 5b, Extended Data Fig. 8b). This rescue was also suggested by the transcriptome-wide similarity between conditional knockout and wild-type or heterozygous controls than to knockouts (Extended Data Fig. 9a, b). However, placental vascularization remained underdeveloped and the conceptuses still died at mid-gestation (Extended Data Fig. 9b). This indicates an essential additional function of Bap1 in the extraembryonic mesoderm compartment that prevents placental labyrinth formation and also results in a yolk sac defect in knockouts and conditional knockouts (Extended Data Fig. 10a). Similarly, Crb2-null embryos could not be rescued by a genetically functional trophoblast lineage (Extended Data Fig. 10b), a result consistent with the lack of phenotype in mutant TSCs. Because the yolk sac phenotype also remained unchanged in conditional knockouts, it can be concluded that the chorio-allantoic placentation defect is due to the crucial role of Crb2 in mesoderm development.

In summary, in vitro and in vivo analysis of three genes whose mutation causes mid-gestational lethality identified two factors (Nubpl and Bap1) with important roles in the proper expansion and differentiation capacity of trophoblast cells. One of these (Nubpl) is indeed causative of the embryonic lethal phenotype at E9.5.

**Discussion**

Systematic mouse knockout phenotyping efforts undertaken so far have excluded the analysis of extraembryonic tissues, most notably the placenta. Ignoring placental defects as a major contributory factor to fetal demise has previously led to several prominent examples of misannotation of gene function, such as for the tumour suppressor Rbl1 and the oncogene Myc. In both cases, subsequent studies have revealed that restoring gene function to the trophoblast lineage could largely rescue the embryonic defects observed. Here, we report a systematic effort to assess the prevalence of placental abnormalities in P14 lethal or sub-viable mouse mutants that survive to at least mid-gestation.

We find a remarkably high percentage of placental abnormalities among these lines, with two-thirds of all P14 lethal strains exhibiting obvious defects. In particular, knockouts that result in mid-gestational
Figure 4 | Determining trophoblast-specific gene function. a, Analysis of Nubpl−/− TSCs grown in self-renewal conditions (day 0) or after differentiation (diff.) for 3 and 6 days. Data are mean ± s.e.m. *P < 0.05; **P < 0.01; ***P < 0.001 (ANOVA with Holm–Bonferroni’s post-hoc test) of n = 3 (wild-type) and n = 5 (Nubpl−/−) individual clones as independent replicates. Specific defects are summarized in the schematic. b, Equivalent analysis for Bap1−/− TSCs of n = 5 (wild-type) and n = 5 (Bap1−/−) individual clones as independent replicates. EPC, ectoplacental cone; GlyT, glycogen cells; rel., relative; SynT, syncytiotrophoblast (layers I and II); TGC, trophoblast giant cells; Prl2c2 is also known as Pfl; Gjb3 is also known as Cx31.

Figure 5 | Dissecting lineage origins of placental phenotypes. a, Schematic representation of the genetic constitutions of embryo (E) and placenta (P) or trophoblast (T) achieved by conditional Sox2-cre-mediated knockout (KO), and corresponding E9.5 embryos of the Nubpl strain. Phenotypes are representative of at least 12 embryos per genotype. HET, heterozygous. b, Immunofluorescence staining of corresponding placentas for MCT4 (marker of syncytiotrophoblast layer II), E-cadherin and basement membrane component laminin (Lam; demarcates fetal blood vessels). Nuclear counterstain with 4,6-diamidino-2-phenylindole (DAPI). Placental defects are representative of at least three independent mutants per genotype.

lethality are almost certainly associated with an abnormal placenta, underpinning the notion that defects in placentation create a bottleneck for developmental progression past mid-gestation[32]. This frequency of placental defects illustrates the hugely underestimated effect of gene mutations on extraembryonic tissues. Given that approximately 25–30% of all mutations cause embryonic lethality, our data suggest that a placental phenotype has gone unnoticed and unreported in hundreds if not thousands of mutant strains.

Many of the genes associated with placental defects in our screen are part of specific functional hubs, such as the L3mbtl2 Polycomb group complex and the tumour necrosis factor-receptor associated factor (Traf2) network, which seem to be of major importance for placental development. Identification of such molecular nodes holds great promise as a way of gaining insights into the causes of placental defects in humans. Consistent with this, at least three of the genes we assessed, TRAF2, PSPH and BAP1 (through its established interaction with ASXL3) have been implicated in the pathophysiology of human pregnancy disorders, many of which have their origin in defective placentation[33–36].

A unique feature of our study is the integrated analysis of both embryo and placenta. This has revealed important co-associations between the occurrence of a placental phenotype and particular defects within the embryo itself, notably affecting neurodevelopment, the heart and the overall vascular system. A placenta–heart axis has been recognized before[37–40], however, we can now identify highly specific pathologies such as a double outlet right ventricle and ventricular septal defects that strongly correlate with the presence of an abnormal placenta. Effects of placental insufficiency on brain development have also been reported[31,41]; our large-scale screen provides strong correlative
evidence to support this developmental co-relationship. By contrast, a systematic effect of the placenta on vascular development, beyond overall haemodynamics\textsuperscript{13}, has not previously been recognized. The importance of our findings may therefore extend not only through the immediate gestational period, but also into postnatal life, and may help to explain how placental insufficiency can have long-lasting consequences on cardiovascular disease risk, outweighing other behavioural factors\textsuperscript{14}.

Taken together, we demonstrate that placental malformations are far more common than previously thought in embryonic-lethal mutations and co-occur specifically with heart, brain and vascular network defects. Our data highlight the importance of including extraembryonic tissues in studies investigating the genetic basis of congenital abnormalities.

**Online Content** Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

**Received 15 February 2017; accepted 23 January 2018. Published online 14 March 2018.**

---

1. Ayadi, A. et al. Mouse large-scale phenotyping initiatives: overview of the European Mouse Disease Clinic (EUMODIC) and of the Wellcome Trust Sanger Institute Mouse Genetics Project. Mamm. Genome 23, 600–610 (2012).

2. de Angelis, M. H. et al. Analysis of mammalian gene function through broad-based phenotypic screens across a consortium of mouse clinics. Nat. Genet. 47, 969–978 (2015).

3. White, J. K. et al. Genome-wide generation and systematic phenotyping of knockout mice reveals new roles for many genes. Cell 154, 452–464 (2013).

4. Adams, D. et al. Bloombury report on mouse embryo phenotyping: recommendations from the IMPC workshop on embryonic lethal screening. Dis. Model. Mech. 6, 571–579 (2013).

5. Dickinson, M. E. et al. High-throughput discovery of novel developmental phenotypes. Nature 537, 508–514 (2016).

6. Rossant, J. & Cross, J. C. Placental development: lessons from mouse mutants. Dev. Biol. 251, 333–336 (1994).

7. Shiratori, Y., Yamamoto, H. & Nikaido, K. Choroideremia gene product affects trophoblast development and placental vascularisation requires the AP-1 component fra1. Genes Dev. 15, 1021–1032 (2001).

8. Whitehead, P. J. The origins of the developmental origins theory. J. Intern. Med. 261, 412–417 (2007).

9. Barker, D. J., Bull, A. R., Osmond, C. & Simmonds, S. J. Fetal and placental size and risk of hypertension in adult life. Br. Med. J. 301, 259–262 (1990).

10. Rossant, J. Development of the extraembryonic lineages. Semin. Dev. Biol. 6, 237–247 (1995).

11. Guillemot, F., Nagy, A., Auerbach, A., Rossant, J. & Joyner, A. L. Essential role of Mash-2 in extraembryonic development. Nature 371, 333–336 (1994).

12. Luo, J. et al. Placental abnormalities in mouse embryos lacking the orphan nuclear receptor ERÎ±. Nature 388, 778–782 (1997).

13. Yamamoto, H. et al. Defective trophoblast function in mice with a targeted deletion of Sox2. Dev. Biol. 229, 1315–1326 (1999).

14. Wang, J., Magier, J., Schier, A. & Magnuson, T. The mouse Pcg gene is essential for Foxg gene repression and extraembryonic development. Mamm. Genome 13, 493–503 (2002).

15. Shi, W. et al. Choroideremia gene product affects trophoblast development and vascularization in mouse extra-embryonic tissues. Dev. Biol. 272, 53–65 (2004).

16. Schreiber, M. et al. Placental vascularisation requires the AP-1 component fra1. Development 127, 4937–4948 (2000).

17. Mohun, T. et al. Defining the Mechanisms of Developmental Disorders (MDMD): a new programme for phenotyping embryonic lethal mice. Dis. Model. Mech. 6, 562–566 (2013).

18. Geyer, S. H. et al. A staging system for correct phenotype interpretation of mouse embryos harvested on embryonic day 14 (E14.5). J. Anat. 230, 710–719 (2017).

19. Karp, N. A., Heller, R., Yaacoby, S., White, J. K. & Benjamini, Y. Improving the identification of phenotypic abnormalities and sexual dimorphism in mice when studying rare event categorical characteristics. Genes Dev. 205, 491–501 (2017).

20. Weneringer, W. J. et al. Phenotyping structural abnormalities in mouse embryos using high-resolution episcopic microscopy. Dis. Model. Mech. 7, 1143–1152 (2014).

21. Tanaka, S., Kunath, T., Hadjantonakis, A. K., Nagy, A. & Rossant, J. Promotion of trophoblast stem cell proliferation by FGfr4. Science 282, 2072–2075 (1998).

22. Murnane, A., Sienert, A. R. & Hemberger, M. Plet1 is an epigenetically regulated cell surface protein that provides essential cues to direct trophoblast stem cell differentiation. Sci. Rep. 6, 25112 (2016).

23. Latos, P. A. et al. Elf5-centered transcription factor hub controls trophoblast stem cell self-renewal and differentiation through stoichiometry-sensitive shifts in target gene networks. Genes Dev. 29, 2435–2448 (2015).
METHODS

Mouse lines. Most mouse lines were generated using the EUCOMM/KOMP knockout first conditional-ready targeted ES cell resource (http://www.mousephenotype.org/about-ikmc/eucomm-program/eucomm-targeting-strategies; targeted trap ‘tm1a” allele and null ‘tm1b” allele). A few lines were generated by CRISPR–Cas9-mediated gene deletion (‘em1” allele). All lines were produced and maintained on a C57BL/6N genetic background at the Wellcome Trust Sanger Institute (http://www.mousephenotype.org/) as part of the DMDD project16. Use of all animals was in accordance with UK Home Office regulations. The UK Animals (Scientific Procedures) Act of 1986 and approved by the Wellcome Trust Sanger Institute’s Animal Welfare and Ethical Review Body. Gene knockout lines were designated lethal if no homozygous mutants were present among a minimum of 28 pups at P14 and sub-viable if their proportion fell on or below 13% of total offspring from heterozygous intercrosses. Corresponding cut-off criteria applied to the designation of sub-viability at E14.5. These ‘DMDD lines’ were assessed at embryonic days E14.5 and/or E9.5, counting the day of the vaginal plug as E0.5. Embryos, placentas and yolk sacs were collected; embryos were processed for HREM imaging3, placenta were fixed in 4% paraformaldehyde (PFA) and yolk sacs were used for genotyping.

For conditional gene ablation in the embryo proper (‘placental rescue”), lines were mated to Flp expressors to generate conditional ‘tm1c” alleles (http://www.mousephenotype.org/about-ikmc/eucomm-program/eucomm-targeting-strategies), and then crossed with Sox2-cre transgenic mice23. Informative crosses were set up between females carrying at least one conditional allele at the locus of interest and heterozygous males that additionally carried the Sox2-cre transgene. Embryos and placentas were collected as before; genotyping was performed on embryonic tail biopsies.

Histology. For histological analysis, at least three mutant and three wild-type placentas from at least two independent litters (with pairs of mutant and wild-type placentas recovered from the same litter if possible) were processed for routine paraffin histology and embedded side-by-side for each strain. No statistical methods were used to predetermine sample size. Placentas of male and female conceptuses were analysed wherever possible. No other randomization is applicable for this study. In all cases, tissue appearance and cellular architecture of the placentas analysed confirmed they were in viable condition even if the associated embryo had been designated as dead or dying. Consecutive 7-μm sections were produced, and alternate sections mounted. A series of sections per block was processed for haematoxylin and eosin (H&E) staining, using a standard protocol (https://dmdmd.org.uk/placental-analysis-protocols/). Sections through the sagittal midline were chosen for imaging, indicated at E9.5 by the remnant of the uterine lumen and at E14.5 by the site of insertion of the umbilical cord. Slides were scanned on a Hamamatsu slide scanner and images deposited at https://dmdmd.org.uk. Phenotypes of placenta were assessed for each strain, blinded for strain viability scores, and recorded by at least two independent investigators. In cases in which all three mutant placentas exhibited a particular abnormality, that defect was scored as a phenotype. In cases in which a defect was unambiguously detected only in two of the initial three placentas analysed, an additional two or three mutant placentas were added to confirm the call. Overall, a phenotype was scored when at least 67% of mutant placentas exhibited that particular abnormality. Criteria for assessing yolk sac morphology encompassed apposition of the visceral yolk sac endoderm and mesoderm layers, and the appearance of blood islands.

Immunostaining and in situ hybridization. To gain a more precise view of the structural defects in mutant placentas, mutant placentas from all lines were stained for E-cadherin (Cdh1) to demarcate the labyrinthine syncytiotrophoblast (as well as parietal giant cells at E9.5) and with isolectin BSI-B4 to outline labyrinthine spongiotrophoblast and glycogen cells. In situ hybridization was used to label the band 3 transcript, reverse reverse with the Ensembl v90 annotation as a search target. In situ hybridization for Tpbe was used to label the spongiotrophoblast and decidua. In situ hybridization for Tpbe was used to label the band 3 transcript, reverse reverse with the Ensembl v90 annotation as a search target.

For immunostaining, sections were deparaffinised in xylene and processed through ethanol series to PBS. Antigen retrieval was performed by boiling in 1 mM EDTA pH 7.2, 0.05% Tween-20 or in 10 mM Na-citrate pH 6.0 buffer followed by blocking in PBS, 0.5% BSA, 0.1% Tween-20. Antibodies used were anti-Cdh1 (1:100; BD Biosciences 6108181), anti-laminin (1:100; Sigma L9393), anti-MCT4 (1:100; Merck Millipore AB3314P) and biotin-conjugated isolectin from Bandeiraea simplicifolia BS1-B4 (1:100; Sigma LS2140). Primary antibodies were detected with appropriate fluorescence or horseradish peroxidase-conjugated secondary antibodies; BSI-B4 was detected with horseradish peroxidase-conjugated streptavidin. Nuclei were counterstained with haematoxylin or DAPI. In situ hybridization for Tpbe was performed using a standard protocol21.

Phenotype data analysis. All genes associated with a placental phenotype in mutant mouse lines were selected for interaction network analysis using esYn (http://www.esyn.org), displaying genetic and physical interactions. Expression data for all genes assessed in mouse mutants was obtained using esyN (http://www.esyn.org), displaying genetic and physical interactions using esyN and poor quality samples were removed. A variance-stabilization transformation was applied to count data for each gene using the R package DESeq2 varianceStabilizingTransformation function48. Principal component analysis was performed on the transformed count data for each gene using the R pcompr function.

© 2018 Macmillan Publishers Limited, part of Springer Nature. All rights reserved.
Data availability. All placental phenotyping data are available at https://dmdd.org.uk. Sequence data were deposited in the European Nucleotide Archive (ENA) under accession ERP023265. All primers sequences are provided. All other data are available from the corresponding author upon reasonable request.

45. Hemberger, M., Nozaki, T., Masutani, M. & Cross, J. C. Differential expression of angiogenic and vasodilatory factors by invasive trophoblast giant cells depending on depth of invasion. Dev. Dyn. 227, 185–191 (2003).

46. Wilson, R., McGuire, C., Mohun, T. & Project, D. Deciphering the mechanisms of developmental disorders: phenotype analysis of embryos from mutant mouse lines. Nucleic Acids Res. 44 (D1), D855–D861 (2016).

47. Hartley, S. W. & Mullikin, J. C. QoRTs: a comprehensive toolset for quality control and data processing of RNA-seq experiments. BMC Bioinformatics 16, 224 (2015).

48. Love, M. I., Huber, W. & Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15, 550 (2014).
Extended Data Figure 1 | See next page for caption.
Extended Data Figure 1 | Potential trophoblast gene function in mutants with placental defect. a, Expression of trophoblast control genes and the 103 DMDD genes in TSCs, TSCs differentiated for 1 or 3 days (d), and in E11.5 placentas. log2-transformed expression values (read counts per million) of RNA-seq data are displayed. Note that all genes associated with a placental phenotype in mutants (labelled in red font) are expressed in trophoblast. b, Frequency of placental defects annotated in mid-gestational lethal mutants (MP: 0011098) as annotated in the MGI database, compared to the findings in DMDD in which 40 out of 41 E9.5–E14.5 embryonic lethal mice were found to exhibit placental abnormalities. c, Left, volume rendered 3D model of the surface of a wild-type embryo, staged as Theiler stage (TS) 23, and coronal section through the volume rendered model. Right, equivalent images of a littermate E14.5 H13−/− embryo, staged as TS21. Note that the models are displayed in identical resolutions. Scale bar, 1 mm. Images are representative of at least five embryos per genotype. d, Network analysis using esyN (http://www.esyn.org) for all DMDD genes identified as causing a placental phenotype in mutants. BAP1 and ASXL3 are known interactors in humans. Red circles identify genes implicated in human trophoblast-based pathologies. The analysis reveals molecular nodes that appear to be of key importance for placental development.
Extended Data Figure 2 | Identification of placental defects by haematoxylin and eosin histology. a, Schematic representation of key stages and cell types in extraembryonic development, complementing Fig. 2c, d. All, allantois; Ch, chorion; Epi, epiblast; ExE, extraembryonic ectoderm; PE, primitive endoderm; SynT-I and SynT-II, syncytiotrophoblast layers I and II; TE, trophectoderm; VE, visceral endoderm. b, Examples of E9.5 placental phenotypes. Dotted lines denote the boundary to maternal decidua; vertical bars denote chorion trophoblast thickness; arrows in the wild-type placenta indicate invagination sites of extraembryonic mesoderm-derived blood vessels into chorionic trophoblast; arrowheads in the Psph−/− placenta denote sites of chorion folding but missing blood vessels; arrowheads in the Dpm1−/− placenta denote overabundant and enlarged trophoblast giant cells. c, Examples of E14.5 placental phenotypes. Red arrows indicate abnormal maternal blood accumulations. Arrows in Traf2−/− and Col4a3bp−/− (including inset) placentas denote fibrotic and/or necrotic areas; arrowheads in Chtop−/− and Pth1r−/− placentas indicate abnormal spongiotrophoblast inclusions. Representative mutant embryo images are also depicted. Images of mutant placentas in b and c are representative of at least three independent mutants per line, see Methods.
Extended Data Figure 3 | Co-association analysis between embryo and placenta phenotypes. 

**a**. Mutant mouse lines were classified into those that exhibit a placental phenotype at E14.5 and those that do not. All embryos analysed by HREM imaging were tagged accordingly to either of these two groups. Enrichment of embryonic phenotype terms in mutant strains with normal or abnormal placentas is shown (dark red denotes fully penetrant phenotype). For brevity, the ‘abnormal’ description has been removed from ontology terms. 

**b**. Significantly enriched embryonic phenotype terms in lines that exhibit an abnormal placenta (see also Supplementary Table 2) versus those with normal placenta. Following hypothesis testing using Fisher’s exact test, adjusting for multiple testing using the Benjamini–Hochberg method, we estimated the magnitude of the abnormal placenta effect. This was determined by calculating independent binomial proportions for the two groups of embryos with normal \( n = 172 \) and abnormal \( n = 69 \) placenta. The percentage difference between groups and the \( P \) values are shown.
Extended Data Figure 4 | See next page for caption.
Extended Data Figure 4 | Specific embryonic defects are significantly correlated with the occurrence of an abnormal placenta. a, Further, detailed co-association statistics between the occurrence of a placental phenotype and specific abnormalities in the embryo proper in DMDD lines. As before, mutant mouse lines were classified into those that exhibit a placental phenotype at E14.5 and those that do not. All embryos analysed by HREM imaging were tagged accordingly to either of these two groups. Significant differences in the frequency of specific embryonic defects was determined between these two groups, and scored for the size of the effect and for its significance. Following hypothesis testing using Fisher’s exact test, adjusting for multiple testing using the Benjamini–Hochberg method, we estimated the magnitude of the abnormal placenta effect. This was determined by calculating independent binomial proportions for the two groups of embryos with normal \((n=172)\) and abnormal \((n=69)\) placenta. The figure shows the differences in the estimated abnormality rates of the two embryo groups, and the extent of the bars represent the 95% Newcombe confidence interval (see Methods). ‘True’ means that these associations are significant, ‘false’ that they fall below the significance threshold. Please note that some terms, such as eye development and growth/size/body region, are probably a consequence of developmental retardation. However, the highlighted terms such as heart, brain and vascular system morphology are definitely based on abnormalities that are not merely due to developmental delay. b, Same analysis as in a but only including those specific embryos whose placentas that were analysed histologically (as opposed to all embryos per strain; \(n=81\) and \(n=41\) embryos having normal and abnormal placenta, respectively). Note that the important and meaningful terms hold up to significance irrespectively. c, HREM image of an example of a massive subcutaneous oedema (asterisk) covering the entire back of a \(Psph^{-/-}\) embryo. Volume rendered 3D model. Axial section through the level of the heart is shown as inlay. Note also the delay in developmental progress. d, Muscular ventricular septal defect (arrowhead) in an \(Atp11a^{-/-}\) embryo. Coronal section through volume rendered 3D model. Axial HREM image is shown as inlay. la, left atrial appendix; lv, left ventricle; pt, pulmonary trunk; ra, right atrial appendix; rv, right ventricle; vs, ventricular septum. Embryo defects shown in c and d are representative of at least three independent mutants.
**Extended Data Figure 5** | **Major routes of TSC differentiation.** Diagram of the main differentiation routes of TSCs, including representative cell type-specific marker genes.
Extended Data Figure 6 | Selection of genes for in-depth analysis of trophoblast contribution to embryonic lethality. a, E9.5 phenotypes of mutant placentas of the three genes (Nubpl, Bap1 and Crb2) chosen for ablation in TSCs, as well as for placental rescue analysis in vivo (Fig. 5, Extended Data Figs 8–10). Black arrows (wild-type placenta) denote fetal blood vessels penetrating into the chorionic ectoderm. Vertical bars denote unpatterned appearance of chorion. Orange arrows indicate empty or fibrotic maternal blood spaces. Images are representative of at least three mutants per line. b, Details of CRISPR design and TSC clone screening strategy for the three selected genes Nubpl, Bap1 and Crb2. All targeted exons were first confirmed to be expressed in trophoblast. RT–qPCR (performed in technical triplicate per clone) and genomic genotyping PCR analysis (performed in duplicate per sample, with results independently confirmed by RT–qPCR data) were performed on individual, single-cell expanded TSC clones to confirm homozygous knockout. Of note, even though splicing may occur across the deleted exon, all CRISPR–Cas9 deletions were designed to result in a premature stop codon. RT–qPCR data are mean ± s.e.m. of n = 3 technical replicates.
Extended Data Figure 7 | Analysis of mutant TSCs for defects in TSC maintenance and differentiation. 

**a**, *Nubpl*−/− TSC clones assessed for additional trophoblast marker genes by RT–qPCR. 

**b**, Additional marker gene analysis on *Bap1*-mutant TSCs. 

**c**, Analysis of *Crb2*−/− TSC clones for a phenotype in stem-cell maintenance (0 days) or during differentiation (3 or 6 days). No significant difference in cell morphology, growth behaviour and gene expression pattern was observed compared to wild-type vector control clones. Data are mean ± s.e.m. *P* < 0.05; **P** < 0.01 (ANOVA with Holm–Bonferroni's post-hoc test).
Extended Data Figure 8 | Placental rescue of Sox2-cre-mediated conditional knockout of Nubpl. 

a, Additional images of Nubpl-mutant embryos showing that a wild-type trophoblast compartment rescues the developmental retardation phenotype and embryonic defects observed in the full knockout at E9.5. At E11.5, Nubpl−/− embryos can still be recovered, whereas complete knockout embryos are not retrievable any more. Images are representative of at least ten independent embryos with the corresponding genotype.

b, Histological analysis of the corresponding placentas at E11.5 shows a complete rescue of the placental defect in conditional knockouts with a genetically functional trophoblast lineage. Sections were stained for MCT4 (a SynT-II marker), E-cadherin (a global SynT marker) and laminin (blood vessel basement membrane marker). Images are representative of three placentas per genotype.
Extended Data Figure 9 | Transcriptomic analysis of placentas from rescue experiments and developmental performance of Bap1 conditional knockouts. a. Principal component analysis of global transcriptomes of E9.5 placentas with the indicated genotype. ‘Res’ refers to placentas from Sox2-cre-mediated conditional knockouts in which the trophoblast lineage remains functional, whereas the embryo is ablated for the gene of interest (E: KO; T: HET). b. Top, E9.5 embryo photos of the depicted genotypes for the Bap1 strain. The embryonic lethality of the complete Bap1 knockout cannot be rescued by a functional trophoblast compartment. Images are representative of at least 12 independent embryos per genotype. Bottom, histological analysis of the corresponding placentas, stained as in Fig. 5b and Extended Data Fig. 8b. Arrows point to partially rescued syncytiotrophoblast loops and some vascular invaginations into the chorionic ectoderm. Yet the vascularisation of the forming labyrinth layer remains under-developed compared to controls. Images are representative of three placentas per genotype.
Extended Data Figure 10 | Analysis of yolk sac morphology in Nubpl, Bap1 and Crb2 mutants and developmental performance of Crb2 conditional knockout. a, Immunofluorescence staining of yolk sacs for E-cadherin (green) and laminin (red) demarcating the visceral endoderm (VE) and basement membrane of the yolk sac mesoderm (YSM), respectively. Bl, blood cells. Bap1 and Crb2 mutants show a defect characterized by the lack of attachment of the two visceral yolk sac layers (arrows). This defect cannot be rescued by the Sox2-cre-mediated conditional knockout, indicating that its cause resides in the extra-embryonic mesoderm lineage. b, Developmental performance of Crb2 knockout and conditional knockout embryos and analysis of placental morphology, equivalent to Extended Data Figs 8b and 9b. No rescue of embryonic lethality or placental defects is observed in the conditional knockouts. Images are representative of at least three independent conceptuses per genotype.
Experimental design

1. Sample size

Describe how sample size was determined.

For histological analysis, at least 3 mutant and 3 wild-type placentas recovered from at least 2 independent litters were processed for paraffin histology and embedded side-by-side in one block for each strain. In the absence of known effect sizes, no statistical method was used to pre-determine sample sizes. Phenotypes of placentas were assessed for each strain, blinded for strain viability scores, and recorded by two (H&E) or three (other types of trophoblast staining) independent investigators. In cases where all 3 mutant placentas exhibited a particular abnormality, that defect was scored as a phenotype. In cases where a defect was unambiguously detected only in 2 of the initial 3 placentas analysed, an additional 2-3 mutant placentas were added to confirm the call. Overall, a phenotype was scored when at least 67% of mutant placentas exhibited that particular abnormality. Reassuringly, the phenotypes were independently confirmed in each of the 4 staining methods (H&E, Tpbpa ISH, E-Cadherin IHC, BSI-B4 lectin staining).

For mutant trophoblast stem cell lines, the number of independent clones analysed is provided for each experiment in the figures (between n=3 and n=6 independent clones for each genotype).

2. Data exclusions

Describe any data exclusions.

Mouse line inclusion criteria: Gene knockout lines were designated lethal if no homozygous mutants were present amongst a minimum of 28 pups at P14, and sub-viable if the proportion of mutant pups fell on or below 13% of total offspring from heterozygous intercrosses. Of these, lines that did not produce recoverable mutant conceptuses at either E9.5 or E14.5 were not analysed as part of the DMDD programme.

No other mouse line or phenotype data exclusions are applicable.

3. Replication

Describe the measures taken to verify the reproducibility of the experimental findings.

Replication was achieved insofar as the phenotype was unambiguously detected in at least 67% of mutant placentas (i.e. either 3/3 or ≥ 4/6). The phenotype was present in mutant placentas recovered from at least two independent litters. Four independent staining techniques, performed on at least 3 wild-type and 3 mutant placentas of each line in parallel, provided further independent replication and validation of phenotype calls. For trophoblast stem cells, analysis of 3-6 independently targeted knockout clones of each genotype ensured the robustness and statistical significance of data.

4. Randomization

Describe how samples/organisms/participants were allocated into experimental groups.

For each mouse line, a random selection of WT and MUT placentas from female and male conceptuses was chosen. Wherever possible, these were selected pair-wise from the same litters, and included both male and female conceptuses. No other randomization is applicable for this study.

5. Blinding

Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

The sample identities (wild-type or mutant) were not blinded per se, but the viability of the mouse line was (which is the important determinant of phenotype for this study). All phenotype annotations were made independently by two (H&E) or three (other types of trophoblast staining) different investigators.

Note: all in vivo studies must report how sample size was determined and whether blinding and randomization were used.
6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

| n/a | Confirmed |
|-----|-----------|
|   | □ □ The exact sample size \( n \) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.) |
|   | □ □ A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly |
|   | □ □ A statement indicating how many times each experiment was replicated |
|   | □ □ The statistical test(s) used and whether they are one- or two-sided |
|   | □ □ Only common tests should be described solely by name; describe more complex techniques in the Methods section. |
|   | □ □ A description of any assumptions or corrections, such as an adjustment for multiple comparisons |
|   | □ □ Test values indicating whether an effect is present |
|   | □ □ Provide confidence intervals or give results of significance tests (e.g. P values) as exact values whenever appropriate and with effect sizes noted. |
|   | □ □ A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range) |
|   | □ □ Clearly defined error bars in all relevant figure captions (with explicit mention of central tendency and variation) |

See the web collection on statistics for biologists for further resources and guidance.

7. Software

Describe the software used to analyze the data in this study.

Adobe Photoshop CS6 for assembly of multiple image captions; Excel for Mac 2011 for RT-qPCR analyses; R packages used are described in Methods section.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a third party.

The majority of mouse lines were generated using the EUCOMM/KOMP knockout first conditional-ready targeted ES cell resource (http://www.mousephenotype.org/about-ikmc/eucomm-program/eucomm-targeting-strategies) and are available through this portal. The few lines generated by Crispr-Cas9 mediated gene deletion (“em1” allele) are available through the Sanger Institute (mouseinterest@sanger.ac.uk). Mutant TS cell lines are available on request directly to the Hemberger lab (myriam.hemberger@babraham.ac.uk). There are no restrictions on the availability of any of the other materials used.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

Antibodies used were anti-Cdh1 (1:100 BD Biosciences 610181), anti-Laminin (1:100 Sigma L9393), anti-MCT4 (1:100 Merck Millipore AB3314P) and biotin-conjugated isolectin from Bandeiraea simplicifolia BSI-B4 (1:100 Sigma L2140). Primary antibodies were detected with appropriate fluorescence or horseradish peroxidase-conjugated secondary antibodies; BSI-B4 was detected with horseradish peroxidase-conjugated Streptavidin. All these antibodies are well-established and have been described in many studies before. Staining specificity was confirmed by isotype controls as well as by secondary-antibody only controls. Further confirmation was the replication of well-established staining patterns (membrane-localised staining for E-Cadherin, basement membrane staining for Laminin, syncytiotrophoblast staining for MCT4, labyrinth and decidual staining for BSI-B4).
10. Eukaryotic cell lines
   a. State the source of each eukaryotic cell line used. Trophoblast stem cell line TS-Rs26, a kind gift from the J. Rossant lab, Hospital for Sick Children, Toronto, Canada.
   b. Describe the method of cell line authentication used. The cell line is authenticated by morphological characteristics, differentiation characteristics and by temporally regulated expression of well-established trophoblast marker genes, that are exclusively expressed in differentiating trophoblast and not in any other cell type.
   c. Report whether the cell lines were tested for mycoplasma contamination. Yes, they were tested and proved negative.
   d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use. None of the cell lines used are listed in the ICLAC database.

11. Description of research animals
    The animal species used in this study was the house mouse, Mus musculus. All knockout mouse lines were produced and maintained on the genetic background of the C57BL/6N strain at the Wellcome Trust Sanger Institute (http://www.mousephenotype.org/) as part of the DMDD project. Use of all animals was in accordance with UK Home Office regulations, the UK Animals (Scientific Procedures) Act of 1986 and approved by the Wellcome Trust Sanger Institute’s Animal Welfare and Ethical Review Body. Heterozygous animals were used for intercrosses to obtain homozygous mutant offspring. Average (mean) age of females was 10.49 wks (age range 5-30 weeks) and of males 14.48 wks (age range 6-40 weeks). Offspring were analysed at P14 to determine whether mutants were obtained at ≤13%, which provided the inclusion criteria for this study. The P14 lethal or subviable lines were analysed at E14.5 and/or E9.5. Male and female conceptuses of each mutant line were analysed.

12. Description of human research participants
    This study did not involve human research participants.