Diverse species-specific phenotypic consequences of loss of function sorting nexin 14 mutations

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Mutations in the SNX14 gene cause spinocerebellar ataxia, autosomal recessive 20 (SCAR20) in both humans and dogs. Studies implicating the phenotypic consequences of SNX14 mutations to be consequences of subcellular disruption to autophagy and lipid metabolism have been limited to in vitro investigation of patient-derived dermal fibroblasts, laboratory engineered cell lines and developmental analysis of zebrafish morphants. SNX14 homologues Snz (Drosophila) and Mdm1 (yeast) have also been conducted, demonstrated an important biochemical role during lipid biogenesis. In this study we report the effect of loss of SNX14 in mice, which resulted in embryonic lethality around mid-gestation due to placental pathology that involves severe disruption to syncytiotrophoblast cell differentiation. In contrast to other vertebrates, zebrafish carrying a homozygous, maternal zygotic snx14 genetic loss-of-function mutation were both viable and anatomically normal. Whilst no obvious behavioural effects were observed, elevated levels of neutral lipids and phospholipids resemble previously reported effects on lipid homeostasis in other species. The biochemical role of SNX14 therefore appears largely conserved through evolution while the consequences of loss of function varies between species. Mouse and zebrafish models therefore provide valuable insights into the functional importance of SNX14 with distinct opportunities for investigating its cellular and metabolic function in vivo.

Mutations in the human Sorting Nexin 14 (SNX14) gene cause spinocerebellar ataxia, autosomal recessive 20 (SCAR20; OMIM 616354)¹. These mutations most often lead to complete loss or truncation of the SNX14 protein, resulting in early onset cerebellar atrophy, ataxia, developmental delay, intellectual disability and coarse facial features, with hearing loss, relative macrocephaly and seizures only reported in some patients¹⁻⁷. SNX14 is ubiquitously expressed among tissues, accounting for the clinically recognisable syndromic presentation characteristic of SCAR20¹⁻⁵. SNX14 belongs to the RGS-PX protein family, which includes SNX13, SNX19 and SNX25⁸. No mutations in these other members have yet been identified as the cause of human diseases. Inside the cell, SNX14 mutations impact both autophagy and lipid metabolism¹⁻³. The most apparent subcellular phenotype is the accumulation of autolysosomes containing lipids¹⁻³. SNX14 is localised to the endoplasmic reticulum membrane via its N-terminal transmembrane domain where it is enriched in proximity to lipid

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difficult to identify and examine particular organs (Fig. 2E). At E10.5, and at a higher frequency at the earlier stage of E10.5 (Fig. 2A; Table S2). However, even at E10.5 the number revealed Purkinje cell loss which is consistent with data from post-mortem tissue from humans with SCAR20,15. In

SNX14 will be essential. The only other vertebrate besides humans reported with a naturally occurring mutation CRISPR-Cas9-mediated non-homologous end joining was used to generate a heterozygous deletion of 571 Snx14 in mice we first constructed a mouse line carrying a deletion in the consequence of loss of gene.

KO/KO mice were ever born (Fig. 2A; Table S2). This suggested that the homozygous Snx14 embryos were visibly smaller with reduced vasculature in the head (Fig. 2C). Additional detailed structural SNX14 is required for viability in mice during the second week of gestation.

Results

SNX14 is required for viability in mice during the second week of gestation. To examine the consequence of loss of Snx14 in mice we first constructed a mouse line carrying a deletion in the Snx14 gene. CRISPR-Cas9-mediated non-homologous end joining was used to generate a heterozygous deletion of 571 nucleotides flanking exon 3 in G9 C57Bl/6J mice (Fig. 1A). The deleted region encompassing exon 3 was confirmed by Sanger sequencing. This was predicted to result in splicing of exon 2 to exon 4, which would cause a frame shift (K114fs +5*) and lead to a null allele (Fig. 1B; Fig. S1). Snx14WT/ko mice were bred for several generations and were found to be viable and fertile. Genotyping with primers flanking this region (Fig. 1B, arrow heads) gave different sized PCR products in Snx14WT/ko and Snx14KO/ko mice (Fig. 1C; Supplemental Fig. S2 for original gel). Homozygous deletion was predicted to result in a complete loss of SNX14 protein which was confirmed by Western blot analysis showing that SNX14 protein could not be detected in Snx14KO/ko mice (Fig. 1D; Supplemental Fig. S2 for original blots).

Following multiple crosses and subsequent pregnancies between heterozygous (Snx14WT/ko x Snx14WT/ko) breeding pairs, no Snx14KO/ko mice were ever born (Fig. 2A; Table S2). This suggested that the homozygous Snx14 mutation might be embryonic lethal. This was confirmed when Snx14KO/ko embryos were found at E12.5 and at a higher frequency at the earlier stage of E10.5 (Fig. 2A; Table S2). However, even at E10.5 the number of homzygotes did not quite reach that predicted from Mendelian ratios, which, along with their appearance, suggested that the onset of embryonic lethality occurs even before this age in some conceptuses. At both E10.5 and E12.5, Snx14KO/ko embryos were found to weigh less than their littermate controls (Fig. 2B). Snx14KO/ko embryos were visibly smaller with reduced vasculature in the head (Fig. 2C). Additional detailed structural investigation show that Snx14KO/ko mice were also notably smaller than their littermate controls at E9.5 (Fig. 2D).

Table 1. Diverse phenotypic consequences resulting from SNX14 mutations in different species.

| Abnormality                  | Human | Dog | Mouse | Zebrafish | Drosophila | Yeast |
|------------------------------|-------|-----|-------|-----------|------------|-------|
| Neurological                 | Yes   | No  | No    | No        | No         | No    |
| Craniofacial                 | No    | No  | Yes   | No        | No         | No    |
| Skeletal                     | No    | No  | Yes   | No        | No         | No    |
| Metabolic                    | No    | No  | Yes   | No        | No         | No    |

*Snz and Mdm1 are homologues of the entire RGS-PX family (SNX13, SNX14, SNX19 and SNX25).

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deletion flanking exon 3 but in C57BL/6N mice, was recently generated by the International Mouse Phenotyping Consorcia at MRC Harwell using a similar methodology to the C57Bl6/J mutant (Supplemental Table S1). This allowed detailed comparison of a comprehensive set of analyses to many other mutant lines on the identical genetic background17. On this strain, embryonic lethality of homozygous mutants was also confirmed at E12.5, prior to the tooth bud stage (Supplemental Table S2). Interestingly, on comprehensive testing of heterozygote animals, the only significant effect detected using the combined SHIRPA and Dysmorphology testing protocol was increased locomotor activity (p = 4.92 × 10–06) (https ://www.mouse phenotype.org/data/genes /MGI:2155664).

**Snx14 mutations in mice result in placental abnormalities.** To investigate the potential cause of the embryonic lethality in Snx14KO/KO mice, individual placentas were collected and sectioned for histology. H&E stained sections revealed a reduced labyrinthine area (Fig. 3A,B). The placental labyrinth is the area pivotal for nutrient and gas exchange, and hence its development and function are critical for embryonic growth and survival18. Defects in the establishment of the labyrinth are linked to fetal growth retardation and, in more severe cases, intra-uterine lethality. Therefore, disruption to this region was further investigated using an antibody with affinity to monocarboxylate transporter 4 (MCT4), in order to observe the syncytiotrophoblast cells in the labyrinthine region. This specific staining revealed a rather extreme paucity of MCT4-stained syncytiotrophoblast cells in Snx14KO/KO placentas, with the phenotype ranging from almost complete absence to profound underdevelopment (Fig. 3C,D; Supplemental Fig. S4). This spectrum of phenotypic variation may explain the range of embryonic stages at which mutant loss was observed. In contrast to MCT4, E-cadherin was not substantially disrupted in Snx14KO/KO placentas (Fig. 3E,F; Supplemental Fig. S4). The expression pattern of both MCT4 and E-cadherin was comparable between Snx14WT/KO and Snx14WT/WT placentas (Supplemental Fig. S4).

**snx14 mutant zebrafish are viable with no overt phenotype arising during embryonic development.** To investigate the consequences of snx14 mutations in zebrafish, we initially used two independent morpholinos to knockdown snx14 in zebrafish according to standard methods19. In complete contrast to the findings reported previously2, at sub-toxic doses (i.e. not associated with necrosis in the head/brain), no morphological defects were observed on a WT background and no motoneuron abnormalities were detected in isl1-gfp + ve transgenic embryo morphants (data not shown). However, it was difficult to draw definitive conclusions from these experiments owing to the potential for hypomorphic knockdown achieved with morpholinos. Therefore, we examined a zebrafish line (sa18413) carrying an ENU-induced point mutation resulting in a premature stop codon in exon 3 (Supplemental Fig. S5). This mutation is predicted to lead to complete loss of function due to truncation of the Snx14 protein (F55*). Splicing of the flanking exons in the event of exon 3 skipping would also result in an out of frame protein with the introduction of a premature stop codon (L44fs + 37*). Homozygous zebrafish were derived from snx14WT/Mut x snx14WT/Mut in crosses at expected Mendelian ratios and no phenotypic abnormalities were observed (Supplemental Fig. S6).
In a previous study of snx14 morphants, embryos at 48hrs were found to display both a reduced head width and eye width, which was considered concordant with the human cerebellar hypoplasia phenotype. Whilst this differed from our preliminary experiments, a possible explanation in these mutant zebrafish might relate to rescue by maternally expressed transcripts encoding the wild-type allele. We therefore excluded this possibility by breeding maternal zygotic (MZ) mutants—snx14<sup>Mut/Mut</sup> zebrafish. The head and eye widths in snx14<sup>Mut/Mut</sup> zebrafish were similar to those measured in both snx14<sup>WT/WT</sup> and snx14<sup>WT/Mut</sup> zebrafish (Fig. 4A; Supplemental Fig. S7).

There were also no remarkable differences in the nervous system as could be observed with antibodies targeting acetylated tubulin and synaptic vesical protein 2 (Fig. 4B).

**Figure 2.** Homozygous Snx14 mutation causes embryonic lethality in mice. (A) Viable Snx14<sup>KO/KO</sup> embryos are not detected at Mendelian ratios at E10.5 and no Snx14<sup>KO/KO</sup> mice were found at P0. (B) Snx14<sup>KO/KO</sup> weighed less than their Snx14<sup>WT/WT</sup> and Snx14<sup>WT/KO</sup> littermates. Bars = Mean ± SD, *p < 0.05, **p < 0.01, one-way ANOVA. (C) Snx14<sup>KO/KO</sup> embryos appear smaller, without clear vascularisation in the head (insets). (D) Surface visualisation of Snx14<sup>WT/WT</sup> and Snx14<sup>KO/KO</sup> embryos with optical projection tomography. (E) Internal visualisation of Snx14<sup>WT/WT</sup> and Snx14<sup>KO/KO</sup> embryos with high resolution episcopic microscopy (HREM).

**Altered lipid profiles in snx14 mutant zebrafish.** It has previously been reported that human SNX14 mutations disrupt neutral lipid metabolism. In line with this, the addition of exogenous fatty acids (FAs) stimulates SNX14 to localize to junctions between the endoplasmic reticulum (ER) and lipid droplets (LDs), indicating a role for SNX14 in FA homeostasis. To monitor FAs in snx14<sup> Mut/Mut</sup> zebrafish, lipids from whole body lysates of 4dpf zebrafish were extracted and total FA lipidomic profiling was conducted. Total FAs from either the neutral lipid fraction (primarily triacylglycerides and cholesterol esters) or phospholipid fraction (primarily glycerolphospholipids) were examined (Supplemental Table S3). Total FAs from neutral lipids were...
elevated in snx14\textsuperscript{Mut/Mut} compared to snx14\textsuperscript{WT/Mut} and snx14\textsuperscript{WT/WT}. Profiling revealed that these included both saturated FAs (16:0) as well as unsaturated FAs (18:1 and 20:4) (Fig. 4C–E). Phospholipid-derived FAs were also similarly elevated in both snx14\textsuperscript{Mut/Mut} snx14\textsuperscript{WT/Mut} compared to snx14\textsuperscript{WT/WT} (Fig. 4F–H). Therefore, loss of snx14 leads to alterations in FA abundance in zebrafish. Similarly, independent studies of Drosophila flies with CRISPR/Cas9 deletion of snx14 homolog snz also exhibited significantly elevated fatty acids as well as elevated triacylglycerides\textsuperscript{14}. Similar studies were not possible in the available mouse embryos due to the variable degenerative nature caused by their early demise.

Zebrafish behaviour. To assess the functional impact of snx14 mutation we monitored larval mutant locomotor activity over multiple days and nights\textsuperscript{20}. snx14 mutants (homozygotes or heterozygotes displayed no differences in their overall activity levels (Supplemental Fig. S8) or in other behavioural metrics (Supplemental Fig. S9) compared to WT, demonstrating that snx14 mutation did not impact upon larval zebrafish baseline locomotor behaviour.

Discussion
In this study we report the impact of loss of function Snx14 mutations in both mice and zebrafish. Genetic mutations in both species have previously been used to investigate similar diseases that share overlapping phenotypes with SCAR20 such as Niemann-Pick disease\textsuperscript{21,22}. However, in this study Snx14\textsuperscript{KO/KO} mice were embryonic lethal before E12.5, while more surprisingly, mutant zebrafish were viable and fertile. The results for both species therefore contrast markedly with each other but also differ significantly from the clinical findings reported in both humans and dogs\textsuperscript{2,15} (Table 1). In this respect, zebrafish resemble, mutant Drosophila with a genetic ablation of snz, the homolog of snx14, which are also viable and survive into adulthood\textsuperscript{14}. This interspecies variation is evident despite evidence that SNX14 has a conserved biochemical role in lipid biogenesis across species from humans to yeast (Table 1). SNX14 therefore provides a paradigm for differential effects of mutation in the same gene across different species.
Figure 4. Constitutive homozygous snx14 mutations do not impact zebrafish morphology at 4dpf but do increase FAs from neutral lipid and phospholipids. (A) Illustration and demonstration of zebrafish eye (E) width and head (H) width measurements of maternal zygotic (MZ) snx14\(^{WT/Mut}\) fish derived from snx14\(^{Mut/Mut}\) female and snx14\(^{WT/Mut}\) male pairs. (B) Maximum projected confocal images of heads (dorsal view) from 4dpf zebrafish embryos either snx14\(^{WT/Mut}\) or MZ-snx14\(^{Mut/Mut}\). Staining employed immunohistochemistry against acetylated tubulin (green), marking axon tracts and SV2 (magenta) marking neuropil areas. (C–E) Relative FA levels from whole body lysates of 4dpf zebrafish. Neutral lipid fraction-derived FA 16:0 (C), FA 18:1(n9) (D) and FA 20:4(n6) (E) were elevated in snx14\(^{Mut/Mut}\) zebrafish compared to both snx14\(^{WT/WT}\) and snx14\(^{WT/Mut}\) zebrafish. (F–H) Phospholipid fraction-derived FA 16:0 (F), FA 18:1(n9) (G) and FA 20:4(n6) (H) were elevated in both snx14\(^{Mut/Mut}\) and snx14\(^{WT/Mut}\) zebrafish compared to snx14\(^{WT/WT}\) zebrafish. \(N = 3\) (Pool of 6 zebrafish in each lysate), circles = individual lysate values, bars = mean, error bars = SD, \(**(p \leq 0.01)\), n.s. \((p \geq 0.05)\), one-way ANOVA.
Mutations usually result in complete loss of SNX14 protein, however truncation or microdeletions have also been investigated in SCAR20 patient derived tissues. Genotype/phenotype correlations are not well established for SCAR20 but there is some evidence for small protein altering mutations resulting in a less severe phenotype than complete loss of SNX14. In the mouse, the CRISPR-induced 571 bp deletion in Snx14 was predicted to result in a frame shift mutation (K114fs + 5*) which led to the loss of detectable SNX14 protein in homozygous mutants. Homozygous mice are therefore equivalent to the majority of SCAR20 patients, who lack functional SNX14 protein. However, unlike SCAR20 patients, homozygous Snx14-KO/KO mice fail during embryonic development. This finding was replicated in a second CRISPR induced deletion, also of exon 3 but on a different genetic background. The likelihood that the lethality resulted from an off-target effect was thus greatly reduced. The finding of embryonic lethality was surprising given that homozygous loss of function mutations in humans, dogs and now zebrafish do not appear to impact on viability before birth. In mice, SNX14 therefore has a critical function from about mid-gestation onward.

SNX13 is the closest mammalian homologue to SNX14, both sharing a similar protein domain structure. However, a single amino acid difference located within the PX domain of the two proteins was shown to dramatically alter the phosphoinositide binding potential, suggesting a likely altered function. However, like SNX14 in this report, loss of SNX13 in mice was previously demonstrated to also result in embryonic lethality at between E8.5 to E13.5. At E10.5, these mice were described as being considerably smaller, having an open cranial neural tube and had defective vascularisation. The embryonic lethality appeared to be primarily due to placental pathology which was observed as a disruption to the formation of the placental labyrinthine layer, with large, undifferentiated and granular trophoblast cells indicative of a disrupted syncytiotrophoblast. In addition, the visceral yolk sac, which has an important role in mediating embryonic nutrition and maternal–fetal exchange, was described as having altered endocytic/lysosomal compartment with increased numbers of autophagic vacuoles. In homozygous Snx14 mutant zebrafish, the labyrinthine layer also appears thinner, with disrupted differentiation of syncytiotrophoblast cells. These abnormalities have the effect of diminishing the surface area that is available for nutrient transfer between the fetal and maternal blood systems and most likely account for the compromised fetal growth observed along with their failure to survive. Although currently, no human phenotype has been associated with SNX13 in humans, it appears that at least in terms of placental biology, SNX14 may share a functional overlap with SNX13.

Placental defects such as these have recently been identified as a leading cause of embryonic lethality in mouse mutants. In their study of 82 mouse lines which were classified as P14 lethal but where embryos could be recovered at either E9.5 or E14.5, 68% were found to have aberrant placental morphology. Many of these genes might not otherwise have been considered as required for normal placental development. An interesting concept in their report, was the integration of both embryo and placenta pathologies through the Deciphering the Mechanisms of Developmental Disorders program study. This analysis identified notable co-associations between placental and embryonic development, particularly affecting neurodevelopment as well as the heart and vascular system in general. It may thus be feasible that this association is similarly followed by SNX14, which was identified primarily as a neurological disorder in both humans and dogs.

The Snx14 mutant mouse line in this study provides an opportunity to monitor SNX14 function in vivo, albeit early in embryonic development and perhaps most crucially for its role in placental biology. To more accurately model pre- and post-natal development of neurological symptoms, it will be necessary to engineer a conditional Snx14 mutant mouse that can be manipulated in a tissue specific fashion. Inducing SNX14 loss via a Cre-loxP mediated recombination event driven by the promoters of neural crest or parkin like expression may be useful to investigate the nervous system and or cerebellum. Alternatively, research tools such as the Tet-on system may also be useful to avoid embryonic lethality.

Unlike in mice, snx14 loss of function in zebrafish did not result in any clear embryonic pathology. Our result contrasts with a previous study that used snx14 targeting morpholinos in zebrafish, which reported reduced eye and optic tectum width in morphants. We detected no discernible impact on eye or brain morphology in 4dpf snx14 morphants, or fish either in our morphants or genetic mutants. Similar discrepancies have previously been reported, particularly when comparing morpholino and genetic mutations. A main concern focuses on off target effects of morpholinos that may be both p53-dependent and/or independent. It has been stressed that genetic mutants should be the standard method to define gene of function from zebrafish and only then can morpholino methods that recapitulate these findings be used as a reliable method of investigation.

Investigating genetic mutant zebrafish also has potential limitations. The early developing zebrafish embryo relies on maternal mRNA expression before initiation of zygotic mRNA. It is possible that the deleterious effects of snx14 targeting morpholinos prevents rescue of a pathology by silencing the maternal expression of the wild type allele in contrast to genetic snx14 mutants. However, this was ruled out here since the same phenotype was observed when the offspring of snx14-KO/Mut males crossed with snx14-Mut/Mut females were investigated. Another possible explanation for where a premature stop codon results in nonsense mediated decay, can be the upregulation of homologous gene sequences that essentially rescue the phenotype. Whilst recent reports show that morphants don’t trigger this pathway, our morphants mirrored the findings in the genetic mutant.

The zebrafish snx14 mutant (F55*) results in a premature stop codon. As no SNX14 antibody was available that reacts with zebrafish SNX14, the consequence of the F55* mutation could not be demonstrated at the protein level. Whilst exon skipping is predicted to result in a truncated protein (L44fs + 37*), it is possible that an alternative start codon might be utilised to translate an N-terminus truncated SNX14 protein. However, a protein translated from the next available downstream start codon would lack the transmembrane domains that have previously been demonstrated as critical for SNX14 localisation to the endoplasmic reticulum membrane. Considering the possible consequences to the likely transcripts, it is most likely that homozygous mutant snx14 zebrafish produce no functional SNX14 protein. This does not appear to affect viability but does manifest through its impact on lipid metabolism.
SNX14 has previously been associated with a role in endocytic/lysosomal/endoplasmic reticulum associated processes1,2,5,6, and mutations result in disruption to normal lipid metabolism9,10. This metabolic defect therefore provides a link to the phenotypic effects seen in different species and may reflect the fundamental underlying cause of the disease potentially explaining the syndromic presentation of SCAR20. Furthermore, both SNX13 and SNX14 are homologues of the single Drosophila gene snz and the yeast gene Mdm1, both of which have also been demonstrated to have a role in lipid metabolism in Drosophila11,12 and yeast13 respectively. Human SNX13 mutations have also been implicated in disrupted lipid metabolism as evidenced by their association with lipid levels in serum31,32.

There are examples of lipid regulating genes that subtly affect zebrafish behaviour. For example, mutation in the lipid regulator gene pitpca1a leads to apparently healthy fish but display increased wakefulness33. It will therefore be valuable to both investigate older snx14 mutant zebrafish e.g. 4-7dpf to determine if a subtle effect, especially on behaviour, might become more obvious with age and to apply further analytical methods such as testing the mutants in a specific balance assay34. Lipid analysis in the Snx14 mouse mutants proved problematic due to the variable onset of embryonic lethality. However, given the emerging theme of disrupted metabolism across species (Table 1), we predict that this may underlie the placental abnormality/embryonic lethality. This will be a valuable aim for future studies that investigate conditional or inducible Snx14 mutations. To complement animal studies, it will be useful to generate various tissue-specific cell types from human patient iPSCs. These will provide valuable additional models to further investigate diverse aspects of this disease.

In summary, we demonstrate here the consequences of constitutive SCAR20-causing Snx14 mutations in two new model organisms, mice and zebrafish. This provides important evidence for species-specific differences, adding to clinical, veterinary and genetic studies in a variety of species. Our results support a fundamental role of SNX14 in metabolism that whilst conserved across species, manifests as diverse phenotypic consequences. This important insight will be valuable for future studies that aim to find therapeutic approaches for SCAR20.

Materials and methods

Generation of the Snx14 mutant mice. The Snx14 mutant mice were generated using CRISPR-Cas9 by the MRC Harwell Institute, Oxfordshire, UK as previously reported35. Studies were licenced by the Home Office under the Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012 (SI 4 2012/3039), UK, approved by the Animal Welfare and Ethical Review Body (AWERB) at MRC Harwell, and performed according to the ARRIVE guidelines. C57BL/6j and C57BL/6N one-cell embryos were injected with Cas9 mRNA and a pool of sgRNA. A 571 bp deletion was generated in C57BL/6j mice and a 585 bp deletion was generated in C57BL/6N/Tc (C57BL/6N) mice using different sgRNA pools (Table S1). Both deletions removed exon 3 of the Snx14 gene. Mice were genotyped by PCR using forward primer 5′-TAGAGATGGGTCTCATGGGC-3′ and reverse primer 5′-CCTCTAGAGGAGATGCTACTACC-3′. The PCR protocol was carried out with an annealing step of 61 °C for 30 s and an elongation step of 72 °C for 1 min. PCR amplicon were analysed by Sanger sequencing (Fig. S1).

Generation of the snx14 mutant zebrafish. An N-ethyl-N-nitrosourea (ENU) mutagenesis induced zebrafish (sa18413) with a truncating G>A point mutation in exon 3 (F55*) of the snx14 gene was obtained from the European Zebrafish Resource Center (EZRC) and raised at 28.5 °C. All work was carried out in accordance with the UK Animal Experimental Procedures Act (1986) under Home Office licences 70/7,892 and 70/7,612. Zebrafish were genotyped by PCR with forward primer GGA AAT ACT GTG AAC AAC TCC TGA and reverse primer ATTGGGCAGCAGGTATTTCTGG. The PCR protocol was carried out with an annealing step of 56 °C for 30 s and an elongation step of 72 °C for 30 s. This yielded a PCR product of 243 base pairs which is digested with restriction enzyme Bcl1 (Promega, R6651) in the presence of the G>A point mutation (i.e. TGATCA). Genotypes were validated by Sanger sequencing (Fig. S2).

Western blot. Protein lysates of tissue isolated from the tail end of mouse embryos at E10.5 were investigated with Rabbit anti-SNX14 (Sigma, HPA017639) as previously reported6.

Mouse placental histology. The placenta was isolated from embryos at E10.5 and examined as reported previously26. Briefly, placentas were fixed in 4% paraformaldehyde and processed for paraffin embedding following standard procedures. Sections were stained with haematoxylin and eosin (H&E) for gross histological assessment. Sections were also stained with antibodies against the syncytiotrophoblast layer II marker mono-carboxylate transporter 4 (MCT4; Merck Millipore AB3314P, used at 1:100) and against E-cadherin (CDH1, BD Biosciences 610,181, used at 1:100), followed by the appropriate fluorescently labelled secondary antibodies. Nuclear counterstaining was with DAPI.

Optical projection tomography. Mouse embryos were collected at E9.5 and fixed overnight in 4% paraformaldehyde at 4 °C. Embryos were cleared and optical projection tomography were performed as previously described37 on a custom-built optical projection tomography (OPT) microscope38. Images were acquired with a pixel size equivalent to 3.35 μm/pixel. 3D slicer was used for analysis and visualisation39.

High resolution episcopic microscopy. Mouse embryos were prepared for high resolution episcopic microscopy (HREM) as reported40. Slice thickness was set at 2.58 μm and the entire embryo was captured in the field of view. E10.5 and E12.5 embryos were imaged with pixel sizes of 2.18×2.18 μm and 2.75×2.75 μm.
Zebrafish behaviour experiments. For each behavioural experiment, zebrafish larvae (4dpf) were transferred to the individual wells of a square welled 96-well plate (Whatman), then each well was filled with 650 µl of fish water. To track each larva's behaviour, plates were placed into a Zebrabox (ViewPoint Life Sciences) set to quantization mode: detection sensitivity: 15; burst: 50 and freezing: 4. Larvae were continuously tracked at 25 Hz for 70 h on a 14 h/10 h light/dark cycle (lights on: 09:00 a.m. to 23:00 p.m.) with constant infrared illumination. Evaporated fish water was replaced each morning between 09:00 and 09:30 a.m. Following each experiment, larvae were euthanised with an overdose of 2-Phenoxyethanol (Acros Organics), and larval DNA was extracted for genotyping using HotSHOT DNA preparation.

Lipidomic extraction and profiling. Zebrafish were collected at 4dpf and snap frozen on dry ice prior to homogenization in methanol/dichloromethane (1:2 v/v). Lipids were extracted using the three-phase liquid extraction method (3PLE) as described. Lipids were then examined using a SCIEX quadrupole time-of-flight (QTOF) TripleTOF 6,600 + mass spectrometer (Framingham, MA, USA) via a custom configured LEAP InfusPAL HTS-xt autosampler (Morrisville, NC, USA). Analyst TF 1.7.1 software (SCIEX) was used for TOF MS and MS/MSALL data acquisition. Data analysis was performed by MarkerView (SCIEX) peak-picking algorithm and interrogated by an in-house script. For further details, please see full report.

Statistics. Data were analysed using Matlab or GraphPad Prism to perform ANOVA.

Data availability
All data generated or analysed during this study are included in this published article (and its Supplementary Information file).

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The authors declare no competing interests.

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