Kif1bp loss in mice leads to defects in the peripheral and central nervous system and perinatal death

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Goldberg-Shprintzen syndrome is a poorly understood condition characterized by learning difficulties, facial dysmorphism, microcephaly, and Hirschsprung disease. GOSHS is due to recessive mutations in KIAA1279, which encodes kinesin family member 1 binding protein (KIF1BP, also known as KBP). We examined the effects of inactivation of Kif1bp in mice. Mice lacking Kif1bp died shortly after birth, and exhibited smaller brains, olfactory bulbs and anterior commissures, and defects in the vagal and sympathetic innervation of the gut. Kif1bp was found to interact with Ret to regulate the development of the vagal innervation of the stomach. Although newborn Kif1bp−/− mice had neurons along the entire bowel, the colonization of the gut by neural crest-derived cells was delayed. The data show an essential in vivo role for KIF1BP in axon extension from some neurons, and the reduced size of the olfactory bulb also suggests additional roles for KIF1BP. Our mouse model provides a valuable resource to understand GOSHS.

Goldberg-Shprintzen syndrome (GOSHS, OMIM 609460) is a rare human condition characterized by intellectual disability, microcephaly, dysmorphic facial features, peripheral neuropathy as well as Hirschsprung disease in which neurons are absent from the distal bowel1–4. GOSHS is associated with recessive mutations in the gene encoding kinesin family member 1-binding protein (KIF1BP; also known as KBP and KIAA1279)2,4,5. Studies of human, mouse and zebrafish have shown that KIF1BP is expressed in nearly all tissues5–7 (www.brain-map.org). Despite the widespread expression of KIF1BP, GOSHS primarily affects the development of neurons and neural crest-derived tissues4,6. However, there is a wide range of phenotypic variations between GOSHS patients, and mesenchymal tissues can also be affected, including urogenital structures, limbs, eyes and heart2–4,8. Most mutations found in GOSHS patients are thought to cause loss of function of KIF1BP, primarily through nonsense mediated mRNA degradation2.

Kinesins (KIF proteins) are a large family of microtubule-based intracellular motors that transport cargo from one part of the cell to another. Cell biological approaches revealed that KIF1BP interacts with a subset of kinesins including KIF1A, KIF1B, KIF1C, KIF3A, KIF13B, KIF14, KIF15 and KIF18A9–11. Studies using cell lines and zebrafish mutants suggest the main role of KIF1BP is to regulate axon extension. Depletion of KIF1BP from PC12 cells and the SH-SY5Y neuroblastoma cell line reduced neurite length12, while overexpression of KIF1BP resulted in increased neurite length in SH-SY5Y cells13 but in reduced axon length in cultured hippocampal neurons14. kif1bp mutant zebrafish exhibit abnormal CNS and peripheral axon outgrowth15,16. However, kif1bp mutant zebrafish have neurons along the entire length of the bowel and thus do not develop a Hirschsprung disease-like phenotype15.

Mutant mouse models have been critical in understanding many developmental diseases. Here we describe the effects of lack of Kif1bp in mice by generating mice in which Kif1bp is disrupted using CRISPR/Cas9. Our
study confirms a role for KIF1BP in axon extension from subpopulations of peripheral and CNS neurons in vivo, but the smaller brain and olfactory bulbs we observed in Kif1bp null mutants suggest additional roles for KIF1BP during nervous system development.

Methods

Mouse strains. Two lines of Kif1bp+/− mice were generated by CRISPR/Cas9 (see below). All experiments were approved by the Anatomy & Neuroscience, Pathology, Pharmacology and Physiology Animal Ethics Committee of the University of Melbourne and all experiments were performed in accordance with relevant guidelines and regulations. Ret+/−/Tgm mice, in which cDNA encoding tau-EGFP-myc (TGM) had been inserted into the first coding exon of Ret15, were mated to Kif1bp+/− mice.

Generation of Kif1bp null mutant mice using CRISPR/Cas. Mice with targeted disruption of Kif1bp were generated by the Monash University Node of the Australian Phenomics Network. To delete exon 1 of mKBP (2510003E04Rik), guide RNA was designed to bind sequences flanking 3′ UTR and intron 1−2. Guide RNA sequences, which were immediately followed by Protospacer Adjacent Motif (PAM) sequences, were gcgacatgtgcgtgtag (forward) and gcgacagagagctgtgagcagctgttgg (reverse) (Fig. 1A). After microinjection and transfer of two-cell stage embryos into a pseudopregnant female, the genotypes of the newborn pups were determined. The genomic regions flanking the gRNA target were amplified by PCR using specific primers: Fwd (5′CAGCCGGAAGGCTCTGTATTC 3′) and Rev (5′TGATTCGAGGCTTAGTGT 3′). Cloning and then sequencing identified two mutant mice where exon 1 was deleted. Although both mice were missing all of exon 1, the deletions were different (Fig. 1B). Hence, after confirmation of transmission of both mutations, two lines of Kif1bp mutant mice were established. Female and male heterozygous mutants for each line were mated.

Genotyping of Kif1bp lines. Genotyping by PCR was performed on DNA extracted from tail tissues of newborn mice, or liver and tail from embryonic mice. Primers used for mKif1bp genotyping were as described above. PCRs were performed using standard PCR protocols with the addition of 5% DMSO in the reaction. Some genotyping of Kif1bp lines was also performed by Transnetx (Cordova, TN, USA). Conditions and primers used to genotype Ret+/−/tgm mice have been described previously18.

Western blot. Protein was isolated from brain of E12.5 embryos generated by crossing mice heterozygous for Kif1bp. Tissue was lysed in RIPA buffer containing protease inhibitors, manually homogenised on ice and cleared by centrifugation at 14,000g at 4 °C for 15min before storing at −20°C. Samples were processed further for SDS-PAGE followed by western blot analysis. Blots were blocked in 5% milk powder and incubated in either rabbit anti-Kif1bp (1:100, kindly supplied by Maria Alves and Robert Hofstra) or mouse anti-E7 Tubulin (1:500, Developmental Studies Hybridoma Bank) at 4 °C overnight with constant agitation. The Kif1bp antibody was a rabbit polyclonal antibody directed against a peptide encompassing amino acids 24–38 of mKif1bp6. After washing in PBS/Tween 20, blots were incubated in either goat anti-rabbit IgG-HRP or goat anti-mouse IgG-HRP (Zymed, both 1:10,000). Bands were detected using chemiluminescence (Amersham ECL, GE Healthcare).

Immunohistochemistry. Wholemount preparations of E12.5 gastrointestinal tract and lungs, E15.5 small intestine, E16.5 diaphragm and external muscle/myenteric plexus of P0 mice were fixed for immunohistochemistry as described previously13,14. For cryosections of the olfactory bulb/nasal cavities and brainstems, newborn mice were injected with ketamine (200 mg/kg) and xylazine (20mg/kg) followed by transectional tissue perfusion with 4% paraformaldehyde. The heads were post-fixed overnight, washed and then cryoprotected. Cryosections of various tissues were processed for immunohistochemistry as described previously19 using the following primary antisera: goat anti-Sox10 (1:200, Santa Cruz, Dallas, Texas, USA), mouse anti-Tuj1 (1:2000, Covance, North Ryde, NSW, Australia), sheep anti-nNOS (1:2000, gift of Dr Piers Emson)16,17, human anti-α-HuC/D (1:5000, gift of Dr Vanda Lennon)18, rabbit anti-NK1 receptor (NK1R) (1:1000, Developmental Studies Hybridoma Bank) and sheep anti-tyrosine hydroxylase (TH) (1:2000, Chemicon), guinea-pig anti-PHox2b (1:1000, gift of Prof. Hideki Enomoto)19, rabbit anti-NK1 receptor (NK1R) (1:1000, Millipore), goat anti-ChAT (1:50, Millipore) and rabbit anti-GABA (1:1000; Sigma). Secondary antisera used were donkey anti-sheep FITC (1:100, Jackson Labs), donkey anti-mouse Alexa594 (1:200, Molecular Probes), donkey anti-guinea pig FITC (1:100, Chemicon) and donkey anti-human Alexa594 (1:500, Jackson Labs). In some sections, nuclei were labelled with DAPI.

Myenteric neuron counts in the distal colon of P0 mice. The number of Hu and nNOS neurons present in stacked confocal images of 393 μm x 393 μm fields of view of wholemount preparations of distal colon were counted manually using Fiji cell counter.

Measurement of migration distance. The distance from the ileocaecal junction to the most distal Sox10+ cell in wholemount preparations of E12.5 colon was measured from projected confocal images using Fiji software as described previously20. The distance from the junction between the stomach and small intestine and the most distal Sox10+ cell was also measured in wholemount preparations of gut from E10.5 mice using the same method.

Measurement of area occupied by vagal fibers in the stomach and TH+ fibers in the duodenum. Wholemount preparations of stomach from E12.5 mice were processed for Tuj1 immunostaining, imaged on a confocal microscope and then the area occupied by Tuj1 vagal branches was measured in projected images using Fiji software. Wholemount E15.5 gut preparations were immunostained for tyrosine hydroxylase (TH), and then tiled confocal images were montaged into a single image. Using Fiji software, the first 2 mm of the duodenum was marked, and the area containing TH+ fibers within the 2 mm zone was measured.
Measurement of commissure thickness. P0 mice were perfusion fixed as described above and the brain processed for paraffin sectioning and Nissl staining. Sections were scanned and measurements taken using CaseViewer software. Both the anterior and posterior branches of the anterior commissure were measured. As many fibres in the anterior commissure of \( Kif1bp^{−/−} \) mice did not cross the midline, the thickness of the anterior
Figure 2. Kif1bp null mutants have delayed ENCC migration, but not distal aganglionosis or significant defects in enteric neurites. (A,B) Wholemount preparations of the external muscle layers of the rectum of newborn Kif1bp+/+ (A) and Kif1bp−/− (B) mice after immunolabeling for the pan-neuronal marker, Hu (red), and the enteric neuron subtype marker, nNOS (green). (C,D) Densities of Hu+ and nNOS+ neurons (mean ± SD) in the myenteric plexus in line 1 of the Kif1bp mutants. There were no significant differences in the densities of Hu+ (C) or nNOS+ (D) myenteric neurons in the rectum (unpaired t tests, p > 0.05). (E,F) Wholemount preparations of colon from E12.5 Kif1bp+/− (E) and Kif1bp−/− (F) mice stained with antisera to Sox10. The most caudal Sox10+ cell in each preparation is shown by an open yellow arrow, the ileocaecal junction is indicated by a red line, and the distance from the ileoacael junction to the most caudal Sox10+ cell by a white dotted line. (G,H) Quantification of the distance between the most caudal Sox10+ cell and the ileoacael junction in E12.5 Kif1bp−/− mice and their littermates (mean ± SD). Because of possible differences in age between litters, the distance from the ileoacael junction to the most caudal Sox10+ cell in each preparation was expressed as a percentage of the mean distance from the ileoacael junction to the most caudal Sox10+ in control (Kif1bp+/+ and Kif1bp−/−) preparations in that litter. The most caudal Sox10+ cell was significantly closer to the ileoacael junction in Kif1bp−/− mice compared to littermates in each line (unpaired t tests, p = 0.0008 for line 1 and p = 0.0002 for line 2). (I) There was no significant difference in the distance between the most caudal Sox10+ cell and the caudal end of the stomach in E10.5 Kif1bp−/− mice and their littermates, and thus there does not appear to be a delay in the entry of vagal neural crest-derived cells into the gut. (J) Kif1bp does not appear to interact with Ret to regulate ENCC migration. The migration of ENCCs in E12.5 Ret+/+; Kif1bp−/− mice was significantly delayed compared to Ret+/+; Kif1bp+/+ mice (ANOVA, p < 0.05), but there was no significant difference between E12.5 Ret−/+; Kif1bp−/− and Ret−/+; Kif1bp+/+ mice (ANOVA). (K,L) The density of Tuj1+ neurites (number of Tuj1+ pixels/area) (K) p = 0.15) and the ratio of Tuj1+ neurons:Sox10+ cells (L) p = 0.08) at the migratory wavefront were not significantly different in E12.5 Kif1bp−/− mice (red squares) from their littermates (blue circles). (M,N) Small intestine of E12.5 mice. The appearance of Tuj1+ neurites in E12.5 Kif1bp−/− mice (N) was similar to that in littermates (M). (O,P) Migratory wavefront of ENCCs in the mid...
colon of E12.5 mice. The number of Tuj1 + neurites at the wavefront was very variable; in some E12.5 Kif1bp<sup>−/−</sup> mice, there were very few neurites at and close to the wavefront (O), while in other E12.5 Kif1bp<sup>−/−</sup> embryos, most Sox10 + ENCCs were associated with a neurite (P). The most caudal Sox10 + cell in each preparation is indicated with a yellow open arrow. All counts were performed blind to genotype.

Skull bone and cartilage staining. Skull bone and cartilage staining was performed using Alizarin Red and Alcian blue as described previously. 21.

Statistics. GraphPad Prism 7.03 was used to analyse data and prepare graphs. All measurements were performed with the researcher blinded to the genotype of the preparations.

Effects of loss of Kif1bp and interactions with Ret in the development of gut innervation.

Intrinsic innervation. Humans with recessive mutations in KIF1BP have GOSHS, and one of the characteristic malformations is Hirschsprung disease. 4 We therefore examined whether P0 Kif1bp<sup>−/−</sup> mice have neurones along the entire length of the colon along the enteric neural crest cell (ENCC) axis (Supplementary Figure 3). We observed that the number of Tuj1 + neurites at the migratory wavefront was very variable, while in other embryos, most ENCCs were associated with Tuj1 neurites (Supplementary Figure 3). We determined that the number of Tuj1 neurites at the migratory wavefront was very variable (ANOVA, p < 0.05). Thus, Kif1bp<sup>−/−</sup> mice do not appear to interact with Ret to regulate ENCC migration.

kif1bp mutant zebrafish exhibit defective axon growth in the peripheral nervous system, including the ENS. ENCCs migrate in close association with neurites of enteric neurons. 24,25 At mid-embryonic ages, most neurites project caudally along the longitudinal axis of the gut. 26,27,28 We used Tuj1 antisera to examine neurites of intrinsic neurons in the gut of E12.5 mice. In the small intestine of both Kif1bp<sup>−/−</sup> and Kif1bp<sup>−/−</sup> mice, many Tuj1 + neurites projected caudally, and the appearance of the neuronal network was not obviously different between genotypes (Fig. 2M). We also examined Tuj1 + neurites associated with ENCCs at the migratory wavefront in the mid-colon of E12.5 mice and quantified the density of Tuj1 staining in the same field of view as the most caudal Sox10 + cell. The number of Tuj1 neurites at the migratory wavefront was very variable between embryos of all genotypes; in some embryos there were very few Tuj1 + neurites associated with the most caudal Sox10 + ENCCs (Fig. 2O), while in other embryos, most ENCCs were associated with Tuj1 + neurites.
Although the four lowest values for the density of Tuj1+ pixels (number of Tuj1+ pixels/area of gut) at the migratory wavefront were all from Kif1bp−/− mice, overall there was no significant difference in the density of Tuj1+ pixels between Kif1bp−/− mice and their littermates (Fig. 2K, unpaired t test, \( p = 0.15 \)). There were also no significant differences in the density of Sox10+ cells close to the migratory wavefront (data not shown, unpaired t test, \( p = 0.4 \)) or in the ratio of Tuj1+ neurites:Sox10+ ENCCs (Fig. 2L, unpaired t test, \( p = 0.08 \)), although the four lowest values for the Tuj1:Sox10 ratio were from Kif1bp−/− mice. Hence we are unable to associate the delay in ENCC migration with defects in neurites close to the migratory wavefront.

Extrinsic innervation of the gut. The axons of sympathetic neurons that innervate the intestines project along intestinal arteries and first enter the small intestine around E15.5 31. The area occupied by tyrosine hydroxylase (TH)+ fibers in the first 2 mm of duodenum in E15.5 Kif1bp+/+ and Kif1bp−/− mice was compared (Fig. 3A–D), and was significantly smaller in Kif1bp−/− mice (Fig. 3E, unpaired t test, \( p = 0.002 \)).

The vagal innervation of the stomach was examined using Tuj1 antisera, which labels intrinsic neurons as well as vagal fibers. At the gastroesophageal junction of E12.5 Kif1bp+/+ mice, large bundles of vagal nerve fibers extended into the stomach and then branched into smaller bundles. The area occupied by branches of the vagus was significantly smaller in E12.5 Kif1bp−/− mice compared to their littermates (Fig. 4A–C, unpaired t test, \( p < 0.0001 \)), and vagal branches were often missing in the proximal stomach of the null mutants (Fig. 4B). The width of the vagus nerve at the gastroesophageal junction was also smaller in E12.5 Kif1bp−/− mice (Fig. 4A,B,D, unpaired t test, \( p < 0.0001 \)). However, the defect was not fully penetrant as the vagal innervation of the stomach of two Kif1bp−/− mice was well within the normal range (Fig. 4C,D). Vagal branches were also missing, smaller or shorter in the stomach of newborn Kif1bp−/− mice (Fig. 4G,H).

Ret null mutant mice do not have any neurons in the small and large intestine, and in the stomach, neurons are only present in the proximal stomach 32,33. In the stomach of E12.5 Kif1bp+/+; Ret+/+ and Kif1bp−/−; Ret+/+

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**Figure 3.** Kif1bp null mutants have delayed growth of sympathetic fibers into the small intestine. Low (A,C) and higher (B,D) magnification images of wholemount preparations of the duodenum of E15.5 Kif1bp+/+ (A,B) and Kif1bp−/− (C,D) mice after immunolabeling for tyrosine hydroxylase (TH, green). (E) The area occupied by TH+ fibers in the first 2 mm of small intestine (mean ± SD) was significantly less in E15.5 Kif1bp−/− mice compared to their littermates (unpaired t test, \( p = 0.002 \)).
TGM mice, Sox10 + cells and Tuj1 + vagal nerve fibers were present throughout the stomach. In E12.5 Kif1bp+/+; RetTGM/TGM and Kif1bp−/−; RetTGM/TGM mice, Sox10 + cells and Tuj1 + vagal nerve fibers were present only in the proximal stomach, and all Sox10 + cells were associated with vagal nerve fibers (Fig. 4E, E'). In 2/4 Kif1bp−/−; RetTGM/TGM mice, vagal nerve fibers were absent from both sides of the stomach, and the only Tuj1 staining was associated with a very small number of intrinsic neurons (Fig. 4F, F'); in the other 2/4 Kif1bp−/−; RetTGM/TGM embryos examined, a small number of short vagal fibers was present on one side of the stomach only. No vaginal innervation of the pancreas was observed in Kif1bp−/−; RetTGM/TGM mice, similar to that observed in Kif1bp−/− mice (J, K).

**Figure 4.** Kif1bp is required for the development of the vagal innervation of the stomach and pancreas, and interacts with Ret. (A, A', B, B'). Wholemount preparations of the stomach of E12.5 Kif1bp+/+ (A, A') and Kif1bp−/− (B, B') mice after immunolabeling for the pan-neuronal marker, Tuj1 (red), and Sox10 (green), which labels all non-neuronal ENCCs. (C, D) The area occupied by Tuj1 + vagal branches (see dotted lines in A, B) and the width of the vagus nerve at the gastroesophageal junction (see horizontal white line in A, B) were quantified. The area occupied by vagal nerve fibers (C) and the width of the vagus nerve (D) were significantly smaller in E12.5 Kif1bp−/− mice compared to their littermates. (E, F). Kif1bp interacts with Ret to regulate the development of the vagal innervation of the stomach. In the stomach of E12.5 Kif1bp+/+; RetTGM/TGM mice, Sox10 + cells and Tuj1 + vagal nerve fibers were present only in the proximal stomach, and all Sox10 + cells were associated with vagal nerve fibers (E, E'). In most Kif1bp−/−; RetTGM/TGM mice, vagal nerve fibers were absent from the stomach, and the only Tuj1 staining was associated with a very small number of intrinsic neurons (F, F'). (G, H) The vagal innervation of the stomach is still defective at birth. Tuj1 staining (G, H) and drawings (G', H') of the same wholemount preparations of P0 wildtype (G, G') and Kif1bp−/− (H, H') mice. In newborn Kif1bp−/− mice, some vagal branches are thinner (green arrow), missing (red arrow) or shorter (purple arrow). (I–K) Wholemount preparations of E12.5 small intestine and pancreas after immunolabeling for Tuj1 (red) and Sox10 (green). Tuj1 + vagal fibers (arrows) are present in the pancreas of Kif1bp+/+; RetTGM/TGM mice (I) and in small numbers in Kif1bp−/−; Ret+/TGM mice (J), but are absent in Kif1bp−/−; RetTGM/TGM mice (K). Note the absence of Sox10 + ENCC within the small intestine of Kif1bp+/+; RetTGM/TGM and Kif1bp−/−; RetTGM/TGM mice (I, K), but Sox10 + ENCC are present within the small intestine of Kif1bp−/−; Ret+/TGM mice (J). The Sox10 + cells (green) associated with the vagal fibers in the pancreas are likely to be Schwann cell precursors.
fibers were observed in the vicinity of the pancreas in double mutants (Fig. 4K), but were present in other phenotypes (Fig. 4I,J). Thus Kif1bp interacts with Ret to regulate the development of the vagal innervation of the gut. In Kif1bp−/−; RetTGM/TGM mice, all Sox10+ cells in the esophagus appeared to be associated with vagal nerve fibers.

**Effects of loss of Kif1bp on the development of the innervation of the lungs and diaphragm.**

Because Kif1bp−/− mice die very shortly after birth and exhibit breathing defects the innervation of the lungs and diaphragm were examined. The number of Tuj1+ (vagal) fibers in the lungs of E12.5 Kif1bp−/− mice was reduced (Fig. 5A,B). However, there were no noticeable differences in the phrenic innervation of the diaphragm of E16.5 Kif1bp−/− mice compared to littermates (Fig. 5C,D).

**Effects of loss of Kif1bp on the brain.**

Brain size and olfactory bulbs. Newborn Kif1bp−/− mice had significantly lower brain weights than their siblings (0.09 ± 0.01 g for Kif1bp−/− mice versus 0.11 ± 0.01 g for their littermates, mean ± S.D., unpaired t test, p = 0.01). Inspection of the brains of newborn Kif1bp−/− mice revealed noticeably smaller olfactory bulbs (Fig. 6A,C). This was confirmed by measuring the area occupied by the olfactory bulbs from photographs of the dorsal aspect of the brain of P0 mice; in both lines of Kif1bp mice, the olfactory bulbs of Kif1bp−/− mice were about 50% smaller than Kif1bp+/+ and Kif1bp+− littermates (Fig. 6E,F, unpaired t tests, p = 0.0001 for both lines). The cortical surface area was also slightly, but significantly, smaller in Kif1bp−/− mice (Fig. 6G,H, p = 0.008 for line 1 and p = 0.048 for line 2). Histological analysis revealed that the
Figure 6. *Kif1bp* null mutants have smaller olfactory bulbs and cerebral cortices. (A,C) Dorsal view of brains of newborn wildtype (A) and *Kif1bp<sup>−/−</sup>* (C) mice. Note the smaller olfactory bulbs (OB) in the *Kif1bp<sup>−/−</sup>* mouse (C). Cx – cerebral cortex; Cb – cerebellum. (B,D) Horizontal sections through the olfactory bulbs of newborn wildtype (B) and *Kif1bp<sup>−/−</sup>* (D) mice stained for haematoxylin and eosin. The layers of the olfactory bulb, including the glomerular layer (Gl), mitral cell layer (Mi) and granule cell layer (GCL) are apparent in the wildtype (B), but not the null mutant (D). Quantification of the size of the olfactory bulbs (E,F) and cerebral cortex (G,H) in both lines of *Kif1bp* mutant mice determined from photographs of the dorsal views of brains. (E,F) In both lines of mice, the olfactory bulb is significantly smaller in area in *Kif1bp<sup>−/−</sup>* mice (unpaired t tests, p = 0.0001 for both lines). (G,H) In both lines, there is also a smaller, but significant, difference in the area of the cortex (p = 0.008 for line 1 and p = 0.048 for line 2). Note that the y axis does not start at 0 for the graphs of cortical area. (I–L) Coronal sections through the olfactory bulb stained using antibodies to GABA (I,J) and tyrosine hydroxylase (TH, K,L). (I) GABA neurons are abundant in the glomerular layer (Gl) and granule cell layer (GCL) in the olfactory bulb of the wildtype mouse. (J) In the *Kif1bp* null mutant, there are only sparse GABA+ neurons in the outer regions of the olfactory bulb (arrows). (K) TH-immunoreactive neurons are present in the glomerular layer (Gl) of the wildtype mouse. (L) TH+ neurons were also present in the outer regions of the olfactory bulb of *Kif1bp<sup>−/−</sup>* mice, but were sparse and did not form a continuous layer.
of newborn Kif1bp−/− mice were not laminated (Fig. 6B,D), while immunohistochemical studies showed that GABA + and TH + neurons were present in the olfactory bulb of Kif1bp null mutants, but were sparse and were only found close to the periphery (Fig. 6L). There was no obvious difference in the appearance of TuJ1 + axons projecting from the olfactory epithelium towards the olfactory bulbs in horizontal sections of P0 mice (Fig. 6M–P).

**Discussion**

We generated Kif1bp null mice using Crispr/Cas9. Both mouse lines generated showed the same phenotype of neonatal death, decreased brain size, including cerebral cortex, smaller and disorganised olfactory bulbs, reduced anterior commissure, delayed enteric neural crest colonisation of the gut and defective vagal and sympathetic innervation of the gut. We did not observe craniofacial abnormalities or HSCR in the mouse model. These latter aspects of the phenotype are similar to kif1bp mutant zebrafish, which displayed reduced axonal growth, but neurons were present along the length of the gut and no craniofacial abnormalities were observed.

Similarities between the mouse model and GOSHs in humans include alterations in brain development, particularly a reduced overall size of the brain. The reduced size of the olfactory bulbs in mutant mice was particularly striking. The olfactory bulbs also lacked normal lamellated organisation, although at least some of the normal neuronal subtypes were present. Moreover, a significant decrease in size of the anterior commissure was observed. Because the two olfactory bulbs are interconnected via the anterior limb of the anterior commissure, the reduction in anterior commissure size may be due to the reduction in olfactory bulb size. Furthermore, the reduced development of the olfactory bulb may decrease output from the olfactory bulb to the olfactory cortex and in turn result in reduced communication between olfactory cortices, which normally communicate via axons in the posterior limb of the anterior commissure. In one reported case of GOSHs, MRI showed an absence of the anterior commissure, however this patient was not genetically confirmed to have GOSHs, and may have had the phenotypically similar but genetically distinct Mowat-Wilson syndrome instead. We were unable to find any reports of defective sense of smell in GOSHs, although smell is not routinely included in neurological assessments, and smell identification tests would be difficult to perform in GOSHs patients, who also have mental retardation. Like GOSHs in humans, most of the defects we observed in Kif1bp−/− mice were not fully penetrant.

Most GOSHs patients have Hirschsprung disease. Kif1bp mutant mice displayed delayed enteric neural crest colonisation of the gut at E12.5, however by birth the entire gastrointestinal tract was colonised and the mice therefore did not display a Hirschsprung disease phenotype. Zebrafish with mutant kif1bp also have full colonisation of the gut. The human gastrointestinal tract is much longer than that of mouse and zebrafish, and hence human enteric neural crest cells must migrate substantially further than in smaller animals. The human gut may therefore be more sensitive to delays in neural crest migration. Differences between human patients and other mouse models of HSCR support this idea. For example, heterozygous mutations of RET can be sufficient to cause HSCR in humans, while RET−/− mice do not have HSCR-like aganglionosis. However, reducing the amount of Ret in mice to approximately one third of wild-type levels does cause HSCR-like aganglionosis in mice, most enteric neural crest-derived cells migrate in close association with neurites. We examined if the delay in migration of enteric neural crest cells in Kif1bp mutants might be due to a reduction in the number of neurites along the migratory pathway. However, there was no significant difference in the amount of TuJ1 staining close to the migratory wavefront in Kif1bp null mutants, and so the mechanism by which Kif1bp regulates enteric neural crest cell migration remains to be determined.
Mice lacking Kif1bp exhibited some phenotypic features that have not been reported in GOSHS patients, including defects in the extrinsic innervation of the bowel. Previous studies have shown that the development of the vagal innervation of the mouse stomach requires netrin-1,43, Slit ligands44, BDNF45, and the transcription factor, Tbx146, while artemin47, NGF48 and netrin-149 play a role in the development of the sympathetic innervation of the gut50. Our data show that Kif1bp is required for the development of the vagal innervation of the stomach.

**Figure 7.** Kif1bp null mutants have defects in the anterior commissure (AC). (A,B,D,E) Nissl stained, coronal sections of P0 mice, at the levels of the genu of the corpus callosum (A,B) and the ventral hippocampal commissure (D,E) showing the AC (red arrows). The anterior AC (aAC) and posterior AC (pAC) are significantly smaller in Kif1bp−/− mice than wild-type mice (C,F) (unpaired t tests, p = 0.0013 and p = 0.0001). (G,H) There was no significant difference in the width of the corpus callosum at the same levels (p = 0.7 and p = 0.69).

**Figure 8.** No obvious defects in the respiratory nuclei in the brainstem of Kif1bp null mutants. Sagittal cryosections of the brainstem of P0 mice immunostained using antisera to ChAT (red), Phox2b (green) and the neurokinin 1 receptor (NK1R, blue). Phox2b staining of the retrotrapezoid nucleus/parafacial respiratory group (RTN/pFRG) and NK1R staining of the pre-Bötzinger complex (pBC) did not reveal any noticeable differences between P0 wild-type mice (top panels) and Kif1bp−/− mice (bottom panels).

Mice lacking Kif1bp exhibited some phenotypic features that have not been reported in GOSHS patients, including defects in the extrinsic innervation of the bowel. Previous studies have shown that the development of the vagal innervation of the mouse stomach requires netrin-1,43, Slit ligands, BDNF, and the transcription factor, Tbx1, while artemin, NGF and netrin-1 play a role in the development of the sympathetic innervation of the gut. Our data show that Kif1bp is required for the development of the vagal innervation of the stomach.
and the sympathetic innervation of the intestine. \textit{kif1bp} mutant zebrafish were reported to have reduced axons in the ENS, although it was not determined whether intrinsic and/or extrinsic neurons were affected. Sensory axonal neuropathy has been reported in one case of GOSHS, but to our knowledge gastric motility and secretion have not been examined in patients with GOSHS. In future studies it would be interesting to examine vagal and sympathetic ganglia in \textit{kif1bp} mutants to determine if the reduced innervation is due to reduced numbers of cell bodies and/or defects in axon projections.

We observed an interaction between \textit{kif1bp} and \textit{Ret} in the development of vagal innervation of the stomach, but not in the colonization of the intestine by neural crest-derived cells. In mice lacking only \textit{Ret}, vagal nerve fibers occupied around one third of the stomach surface, whereas in mice lacking both \textit{Ret} and \textit{kif1bp}, there were almost no vagal nerve fibers in the stomach or projecting to the pancreas.

An incidental observation of our study was that in \textit{Ret}−/− mice, almost all of the Sox10 + cells, which are only present in the proximal stomach, were associated with vagal nerve fibers. Recent studies have shown that peripheral glial stem cells (also called Schwann cell precursors) are the source of parasympathetic neurons and sub-populations of enteric neurons and adrenal chromaffin cells. Our data support a very recent study showing that enteric neurons in the esophagus and stomach arise from Schwann cell precursors that navigate into the esophagus and stomach along the vagus nerve.

In the current study, loss of \textit{kif1bp} only affected a sub-population of axons in the peripheral and central nervous systems of mice. For example, we found no evidence that the axons of enteric neurons, the phrenic nerve innervating the diaphragm or the corpus callosum were affected in \textit{kif1bp} null mutants. The mechanism by which \textit{kif1bp} affects axonal outgrowth and transport has been examined in several studies. A yeast two-hybrid screen using an E11 mouse cDNA library showed that KIF1BP binds to microtubule-associated proteins, and in human fibroblasts, KIF1BP was reported to co-localize and interact with α-tubulin and F-actin. Moreover, recent studies have demonstrated that KIF1BP binds to and regulates a variety of kinesin motor proteins involved in microtubule cytoskeletal organization and neuronal cargo transport. KIF1BP has been shown to bind KIF1B, SCG10, KIF1A, KIF1C, KIF3A, KIF13B, KIF14, KIF15 and KIF18A, however no association was detected between SCG10 and KIF1BP in the latter study. These members of the kinesin superfamily are from different subfamilies, which have different functions and can interact with different cargo types. As KIF1BP can bind to multiple different members of these subfamilies, it is possible that the differential effects of loss of KIF1BP on axon projections from different neuronal populations might reflect differences in the expression of particular KIFs by different neuron types.

Unlike GOSHS patients, \textit{kif1bp} null mutant mice died within several hours of birth. Premature death was associated with respiratory failure, as indicated by cyanosis and hypoxic gasping. It is possible that respiratory failure is linked to reduced vagal innervation of the lungs. Vagal feedback linked to the Hering-Breuer reflex of pulmonary stretch receptors is critical for neonatal breathing, and vagotomy triggers acute respiratory failure in neonates. Thus reduced vagal innervation of the lung may be a major contributing factor for respiratory failure observed in \textit{kif1bp}−/− mice. Although critical respiratory centers and motor nuclei in the brainstem appear to be normally developed and the diaphragm is innervated in \textit{kif1bp} null mutants, we also cannot rule out the possibility of defects in the connectivity of the neural circuitry underlying respiration. It would be interesting to restore \textit{kif1bp} only in the nervous system to determine if neonatal lethality is rescued.

To date, the main cellular functions identified for KIF1BP are to regulate axon growth and synaptic vesicle transport. However, our data showing reduced olfactory bulb and brain size strongly suggest that KIF1BP must also play an essential role in survival, proliferation and/or neuronal differentiation during development of some parts of the nervous system.

In conclusion, we have generated the first mouse model of GOSHS, which exhibit defects in the development of subpopulations of CNS and peripheral neurons. It is not surprising that mutations that affect general developmental neurobiological processes such as axon extension (this study) and synapse development that underlie autism affect both central and peripheral neurons.

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