Multiple congenital malformations of Wolf-Hirschhorn syndrome are recapitulated in Fgfrl1 null mice

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SUMMARY

Wolf-Hirschhorn syndrome (WHS) is caused by deletions in the short arm of chromosome 4 (4p) and occurs in about one per 20,000 births. Patients with WHS display a set of highly variable characteristics including craniofacial dysgenesis, mental retardation, speech problems, congenital heart defects, short stature and a variety of skeletal anomalies. Analysis of patients with 4p deletions has identified two WHS critical regions (WHSCRs); however, deletions targeting mouse WHSCRs do not recapitulate the classical WHS defects, and the genes contributing to WHS have not been conclusively established. Recently, the human Fgfrl1 gene, encoding a putative fibroblast growth factor (FGF) decoy receptor, has been implicated in the craniofacial phenotype of a WHS patient. Here, we report that targeted deletion of the mouse Fgfrl1 gene recapitulates a broad array of WHS phenotypes, including abnormal craniofacial development, axial and appendicular skeletal anomalies, and congenital heart defects. Fgfrl1 null mutants also display a transient foetal anaemia and a fully penetrant diaphragm defect, causing prenatal and perinatal lethality. Together, these data support a wider role for Fgfrl1 in development, implicate Fgfrl1 insufficiency in WHS, and provide a novel animal model to dissect the complex aetiology of this human disease.

INTRODUCTION

Wolf-Hirschhorn Syndrome (WHS) is a contiguous gene disorder that results in craniofacial defects, microcephaly, mental retardation, congenital heart defects, problems in the development of the brain, the eyes, the ears, the teeth, the hands and the feet, and other malformations. WHS is estimated to occur in about one in every 20,000 births (Maas et al., 2008). Correlations between the highly variable phenotypes of patients with WHS and their genotypes show that the disease is generally caused by deletions of the short arm of chromosome 4 (4p) (Bergemann et al., 2005). Approximately 20% of cases carry chromosomal deletions that are restricted to 4p16.3, but larger deletions are more common and can extend as far as 4p14.

The comparison between interstitial and 4p terminal deletions resulted in the identification of two WHS critical regions (WHSCR-1 and -2) that are approximately 2 Mb from the telomeric end of the chromosome, and two genes located at this site have since been referred to as the Wolf-Hirschhorn syndrome candidate 1 (WHSC1) and 2 (WHSC2) genes. Haploinsufficiency of WHSC1 is considered to be responsible for the typical craniofacial phenotype (‘Greek helmet face’) that is present in almost all WHS patients, but mouse models for WHS show that deletions targeting the WHSCRs on the mouse chromosome do not recapitulate the classical WHS defects (Näf et al., 2001). Instead, the fibroblast growth factor receptor-like 1 (FGFRL1) gene, located at 4p16.3, has been implicated recently as an important candidate gene contributing to the craniofacial phenotype of a WHS patient carrying a small telomeric deletion in chromosome 4 that included the Fgfrl1 locus but left the WHSCRs unaffected (Engbers et al., 2009). A role for Fgfrl1 in human craniofacial development is supported further by the characterisation of a Fgfrl1 mutation in a patient with craniosynostosis (Rieckmann et al., 2009). Small telomeric deletions of chromosome 4 that exclude the WHSCRs but include Fgfrl1 have also been associated with intra-uterine growth retardation and short stature in WHS patients (Maas et al., 2008). As Fgfrl1 is preferentially expressed in skeletal tissues and is prominent in the primordia of the maxillae, the mandibles and the permanent cartilage of the trachea and nose during late stages of mouse development (Trueb and Taeschler, 2006; Trueb et al., 2003), these data collectively point towards a potential role for Fgfrl1 in craniofacial development and suggest that haploinsufficiency of Fgfrl1 in humans with 4p16.3 deletions may contribute to the WHS craniofacial phenotype.

The fibroblast growth factor (FGF) family comprises a complex signalling system involving 22 different ligands in humans and mice that interact with tissue-specific splice variants of four different FGF receptors (FGFRs) to coordinate a wide range of cellular and developmental processes (Bottcher and Niehrs, 2005; Thisie and Thisie, 2005). The independent identification of the Fgfrl1 protein by several research groups added a potential component to the FGF signalling system (Kim et al., 2001; Sleeman et al., 2001; Wiedemann and Trueb, 2000; Wiedemann and Trueb, 2001). The predicted extracellular domain of Fgfrl1 is highly related to the FGFRs, which are cell surface receptor tyrosine kinases with three extracellular immunoglobulin-like domains and a heparin-binding sequence, but Fgfrl1 lacks the intracellular protein tyrosine kinase domain that is essential for FGF signal transduction. Although Fgfrl1 might not directly activate an intracellular signalling cascade, in vitro assays show that Fgfrl1 interacts with FGF ligands and heparin, and thus may function as a decoy receptor in the FGF signalling pathway (Sleeman et al., 2001; Trueb et al., 2003). A single Fgfrl1 gene was found in mammalian (Sleeman et al., 2001), bird (Trueb et al., 2003) and amphibian (Hayashi et al., 2004) species, as well as in the cephalochordate Brachiopterus floridensis (Beyeler and Trueb, 2003).
suggesting that the Fgfrl1 gene predates the two genome duplications that occurred early in vertebrate evolution. In fish species such as zebrafish and *Fugu rubripes*, two copies of the Fgfrl1 gene can be found (Trueb et al., 2005), which is in line with the additional genome duplication during the evolution of ray-finned fish. Functional studies in zebrafish provided in vivo evidence for an essential role of Fgfrl1 in vertebrate craniofacial skeletal development (Hall et al., 2006). Depletion of zebrafish *fgfrl1a* and *fgfrl1b* results in a craniofacial phenotype, including malformations of the lower jaw and developmental inhibition of the cartilage formed by the branchial arches, and the authors propose that *fgfrl1a* is required for FGF signal transduction to promote gill cartilage formation. Fgfrl1-deficient mice, generated by Baertschi et al. (Baertschi et al., 2007), unexpectedly did not reveal any defects in skeletal development. Instead, these Fgfrl1-deficient mice display a prominent diaphragm defect and fail to inflate their lungs after birth, causing perinatal lethality.

To facilitate the analysis of Fgfrl1 function in more detail, we generated an Fgfrl1 mutant allele, targeting different exons from those described by Baertschi et al. (Baertschi et al., 2007). The Fgfrl1 null mice display a broad array of phenotypes, including cardiac abnormalities and skeletal malformations. Defects in cardiac development and foetal anaemia probably caused the prenatal lethality that was observed in a subpopulation of Fgfrl1+/- mutants. The diaphragm phenotype described by Baertschi et al. (Baertschi et al., 2007) was fully penetrant in the Fgfrl1+/- mice that develop until term and, together with the defects in cardiac and skeletal development, caused perinatal lethality. These congenital malformations underscore the crucial function of Fgfrl1 in embryonic development. The significant overlap of the phenotype of Fgfrl1 null mice with clinical WHS presentations confirms a central role for FGFR1 in the human syndrome and implicates a gene outside of the WHSCRs in the WHS phenotype. Fgfrl1 null mice thus provide a model to study FGFR1 activity in FGF signalling and to dissect the complex aetiology of WHS.

**RESULTS**

**Developmental expression of Fgfrl1**

Expression of Fgfrl1 has been well described by Trueb et al. (Trueb et al., 2006) for 15- to 17-day-old mouse embryos, but to evaluate an earlier role for this receptor we studied Fgfrl1 expression by whole-mount in situ hybridisation during early developmental stages. At embryonic day (E)10.5, a wide range of tissues expressed Fgfrl1 including brain, cranial placodes, pharyngeal arches and somites (Fig. 1A). A cross section through the otic vesicle, the first two pharyngeal arches and the nasal pit showed Fgfrl1 expression in the cranial placodes and in a subepithelial compartment of the pharyngeal arches (Fig. 1B). Fgfrl1 was also expressed at a medial site of the hindbrain and expression in the central nervous system extended from the brain to the neural tube (Fig. 1C). The heart was another prominent site of Fgfrl1 expression at E10.5 (Fig. 1D). Transverse sections confirmed a very specific expression pattern of Fgfrl1 in the endocardial cushions of the atrioventricular canal (Fig. 1E) and the cardiac outflow tract (Fig. 1F).

**Fgfrl1 null mice display both prenatal and perinatal lethality**

To delineate the function of Fgfrl1 in embryonic development and its potential role in WHS, we generated mice carrying *Fgfrl1flox*, in which the DNA segment that includes exons 3 to 7 was flanked by *loxP* sites (Fig. 2A-C). *Fgfrl1flox/+* mice, as well as recombined *Fgfrl1+/lox* heterozygotes, were viable and fertile with no discernible phenotype. Quantification of Fgfrl1 mRNA levels in E12.5 *Fgfrl1flox/+* embryos confirmed the absence of Fgfrl1 expression, indicating that the recombined allele is a true null allele (Fig. 2D). Fgfr3, Whsc1 and Whsc2 are located within the WHSCRs and have been linked to WHS characteristics (Bergemann et al., 2005), but mRNA levels of these genes showed no significant differences between wild-type and *Fgfrl1+/−* embryos. This excludes the possibility that changes in the expression levels of these WHSCR genes contribute to the mutant phenotype. *Fgfrl1−/−* embryos were still present at the expected Mendelian ratio at age E18.5 (Table 1). At E14.5 no gross...
morphological distinction was detected between Fgfrl1<sup>−/−</sup> foetuses and their wild-type littermates (Fig. 3A,B), but at E16.5 a subpopulation of the Fgfrl1<sup>−/−</sup> embryos suffered prenatal death, displaying developmental retardation and defective formation of the vascular system (Fig. 3C-E). The vascular defect was also evident in the yolk sac (Fig. 3F,G). Mildly affected Fgfrl1<sup>−/−</sup> embryos could be distinguished from wild-type littermates because of their dome-shaped cranium and short stature (Fig. 3C,D). By E18.5, the subpopulation (approximately 40%) of severely affected homozygotes had completely deteriorated, whereas the Fgfrl1<sup>−/−</sup> embryos that remained at age E18.5 developed until term (Fig. 3H). Surviving Fgfrl1<sup>−/−</sup> neonates were smaller than their wild-type littermates and showed signs of severe respiratory distress, dying immediately after birth or surviving for only several hours. Histological analysis of the lungs of Fgfrl1<sup>−/−</sup> embryos at E18.5 did not reveal any morphological defects compared with wild-type littermates (data not shown) but, similar to previous findings by Baertschi et al. (Baertschi et al., 2007), Fgfrl1<sup>−/−</sup> embryos displayed a severe diaphragm phenotype (Fig. 3I,J) that caused perinatal lethality.

To determine whether the variable expressivity of the Fgfrl1<sup>−/−</sup> phenotype was affected by gender, we examined the sex distribution among the severely affected Fgfrl1<sup>−/−</sup> mutant embryos and those Fgfrl1<sup>−/−</sup> embryos that developed until term (Table 2). Near-equal distributions of males and females in both mutant subpopulations indicated that the variable expressivity of the Fgfrl1<sup>−/−</sup> phenotype is not related to gender.

**Skeletal malformations in Fgfrl1 null mice**

Previous studies reported that Fgfrl1 is primarily expressed in cartilaginous tissues and bone primordia (Trueb and Taeschler, 2006; Trueb et al., 2003). Since craniofacial features are a common

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**Fig. 2. Targeting of Fgfrl1 by homologous recombination to produce the Fgfrl1 null allele.** (A) Schematic representation of the Fgfrl1 locus, targeting construct and Fgfrl1 null allele after Cre-mediated recombination of the targeted locus. Coding exons are depicted as open boxes and the 3’ and 5’ UTRs (untranscribed regions) are shown as black boxes. The FRT-flanked PGK<sup>neo</sup> cassette is depicted as a grey box. LoxP sites in the targeting construct flank exons 3-7 and the PGK<sup>neo</sup> cassette, which are removed after Cre-mediated deletion. Left arm and right arm external probes were used for screening embryonic stem (ES) cells to distinguish the endogenous allele from the targeted allele. Arrows indicate the position of the PCR primers (P1, P2, P3) used for genotyping. (B) Southern blot analysis using the left arm probe on ES cell genomic DNA after SpeI digest, indicating the wild-type (18.7 kb) and the Fgfrl1<sup>flox</sup> allele (8.7 kb) in successfully targeted clones. (C) PCR analysis of embryos derived from matings between mice that are heterozygous for the Fgfrl1<sup>−/−</sup> null allele, showing the 245 bp wild-type band and the 310 bp band of the Fgfrl1<sup>−/−</sup> null allele. (D) Relative quantification of mRNA levels by RT-PCR for Fgfrl1, and the WHSCR genes Fgfr3, Whsc1 and Whsc2 in E12.5 embryos. Values are shown as fold induction in Fgfrl1 mutants relative to wild-type littermates following normalisation to Gapdh expression (mean±s.e.m.). ***P<0.01.
A hallmark of all WHS patients and a large variety of skeletal anomalies are found in up to 70% of individuals with WHS (Battaglia et al., 2001), we investigated skeletal development in those Fgfrl1 null mice that develop until term. Alcian Blue and Alizarin Red S staining of Fgfrl1−/− E18.5 mutants revealed hypoplasia of all skeletal elements, including shortened axial and appendicular skeletons, malformed vertebrae, a small pelvic girdle and a small rib cage, in line with typical WHS skeletal phenotypes (Fig. 4A). At E18.5, the distance between the growth plates of the long bones in Fgfrl1−/− mice was reduced by 19-26% compared with in wild-type littermates (Table 3). The skeleton of Fgfrl1−/− mice also displayed an unusual large gap between the atlas and the occipital bone, in some cases resulting in the bulging of the spinal cord that was evident upon dissection. Closer examination of the head revealed an anterior-displaced foramen magnum in combination with severe hypoplasia and delayed fusion of the sphenoid, basisphenoid, and basioccipital bones that make up the cranial base of the skull (Fig. 4B). A dorsal view of the head showed that the calvarial elements in Fgfrl1−/− mice appeared thin, with a defect in suture closure (Fig. 4C). Midfacial hypoplasia, together with the round-headed appearance and an anterior shift of the foramen magnum in Fgfrl1−/− mice, was reminiscent of the achondroplasia-like phenotype in transgenic mice that express a constitutively active mutant of the MAP kinase kinase MEK1 in chondrocytes (Murakami et al., 2004). Forebrain development was also severely affected in Fgfrl1−/− mice (Fig. 4D,E), consistent with the observation that forebrain defects, mental retardation and facial dysgenesis, which are common in WHS and other subtelomeric deletion syndromes, are often genetically linked (Bergemann et al., 2005). Other phenotypes that overlap with WHS skeletal anomalies (Magill et al., 1980) included a delay in the ossification of the cervical vertebrae (Fig. 4F,G) and abnormal sternal ossifications (Fig. 4H,I) in association with a reduced thoracic diameter (Fig. 4A).

Fgfrl1 is expressed in the pharyngeal arches of mice (this study), zebrafish (Hall et al., 2006) and Xenopus (Hayashi et al., 2004). In zebrafish, fgfrl1a and fgfrl1b are essential for craniofacial development and, in particular, for the formation of gill cartilage derived from the branchial arches (Hall et al., 2006). Laryngeal

<table>
<thead>
<tr>
<th>Genotype</th>
<th>E14.5 (15 litters)</th>
<th>E16.5 (17 litters)</th>
<th>E18.5 (19 litters)</th>
<th>NB (28 litters)</th>
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<tbody>
<tr>
<td>Wild type</td>
<td>32 (26%)</td>
<td>35 (24%)</td>
<td>35 (24%)</td>
<td>55 (30%)</td>
</tr>
<tr>
<td>Heterozygous</td>
<td>56 (46%)</td>
<td>74 (51%)</td>
<td>77 (53%)</td>
<td>117 (63%)</td>
</tr>
<tr>
<td>Homozygous</td>
<td>33 (27%)</td>
<td>37 (25%)</td>
<td>33 (23%)</td>
<td>14 (7%)</td>
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<tr>
<th>Severe</th>
<th>Mild</th>
<th>Severe</th>
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<tr>
<td>16</td>
<td>21</td>
<td>13</td>
<td>20</td>
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Fig. 3. Comparison between Fgfrl1 wild-type (+/+) mice and homozygous mutant (−/−) mice. Wild-type (A) and Fgfrl1−/− (B) embryos at age E14.5 showed no gross morphological differences. Wild-type (C) and Fgfrl1−/− (D,E) embryos at age E16.5. At this stage, a subpopulation of Fgfrl1−/− embryos (E) was severely affected, suffering prenatal lethality and showing developmental retardation. Mildly affected Fgfrl1−/− embryos (D) display a short stature and a dome-shaped cranium. Wild-type (F) and severely affected Fgfrl1−/− (G) embryos at age E16.5 within their yolk sac. Whereas the yolk sac of the wild-type embryo (F) shows a prominent vasculature, the yolk sac of the Fgfrl1−/− embryo (G) lacks a clear vasculature and is devoid of blood. (H) Wild-type and mildly affected Fgfrl1−/− embryos at age E18.5. The Fgfrl1−/− embryos at this stage maintain the typical cranial and short stature phenotype. Comparison between the diaphragm from a wild-type (I) and Fgfrl1−/− (J) embryo at age E18.5 shows that the lumbar (arrows) and sternal (arrowhead) muscle portions of the diaphragm were not present in Fgfrl1−/− embryos and that the remaining costal portions of the diaphragm muscle were very thin in these embryos compared with in their wild-type littermates. The crural muscles (asterisk) in Fgfrl1−/− mice were also smaller compared with control animals and confirm the overall defect in diaphragm development. Bars, 1 mm.
Table 2. Sex distributions among Fgfrl1–/– mice

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<tr>
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<th>Male</th>
<th>Female</th>
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<tr>
<td>Severely affected mutants</td>
<td>8 (53%)</td>
<td>7 (47%)</td>
</tr>
<tr>
<td>Mildly affected mutants</td>
<td>12 (44%)</td>
<td>15 (56%)</td>
</tr>
<tr>
<td>Total number of Fgfrl1–/– mice</td>
<td>20 (48%)</td>
<td>22 (52%)</td>
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cartilage elements that express Fgfrl1 in mice (Trueb and Taeschler, 2006) also derive from the pharyngeal arches and were affected in the Fgfrl1–/– background. The hyoid bone was small and often incompletely ossified, and the thyroid and cricoid cartilages were significantly reduced in size (Fig. 4J,K). The affected laryngeal cartilage elements in Fgfrl1–/– mice also provide an explanation for the problems in swallowing and speech development in infants with WHS (Battaglia et al., 2001) and, in combination with the diaphragm phenotype, might contribute to respiratory distress (van Dooren et al., 2004).

**Fgfrl1 is essential for the formation of cardiac valves and ventricular septation**

The defective blood distribution in severely affected Fgfrl1–/– embryos and their yolk sac led us to focus on the development of the cardiovascular system. At E12.5, cardiac development in Fgfrl1–/– embryos appeared unaffected and the yolk sac had a normal vasculature and blood distribution, but by E14.5 most Fgfrl1–/– embryos showed a defect in the septation of the ventricles, which normally completes around this stage (Fig. 5A,D). Expression of Fgfrl1 in the endocardial cushions of the outflow tract and atioventricular canal at E10.5 (Fig. 1) suggested a role for Fgfrl1 in valvulogenesis. Indeed, the leaflets of both the atioventricular (Fig. 5B,E) and semilunar valves (Fig. 5C,F) were thickened in Fgfrl1–/– embryos compared with in wild-type littermates. These defects persisted in E18.5 Fgfrl1–/– embryos that developed until term (Fig. 5G-L). Ventricular septation defects and cardiac valve-related congenital heart defects, such as pulmonary stenosis and aortic insufficiency, are characteristic for many WHS patients (Battaglia et al., 2001) and were prominent in Fgfrl1–/– embryos (Table 4). Notably, other cardiac defects related to abnormal outflow tract remodelling, such as persistent truncus arteriosus or aortic arch anomalies, were not found in Fgfrl1–/– embryos.

**Fgfrl1 deficiency causes foetal anaemia**

At E12.5, the exchange of oxygen between maternal and foetal blood is established in the labyrinth layer of the placenta when the foetal liver starts producing blood that formerly had been provided by the yolk sac blood islands. Although placental insufficiency could be a primary cause of prenatal lethality in Fgfrl1–/– mice, histological analysis of E9.5 and E10.5 placenta from both wild-type and Fgfrl1–/– mice showed a proper chorioallantoic fusion in all mice, and at E12.5, the vasculature in the allantois of all Fgfrl1–/– mice displayed normal branching in concert with the formation of the chorionic villi (data not shown). By E14.5, there was no gross morphological difference in placental structures between wild-type and Fgfrl1–/– mice (Fig. 6A,B), but E16.5 Fgfrl1–/– embryos that suffered prenatal lethality showed a clear defect in the placenta (Fig. 6C). The labyrinth layer of the placenta in these mutants was highly disorganized, whereas the placenta in those Fgfrl1–/– mice that develop until term did not show any gross morphological defects (Fig. 6D-F). The placental defects in E16.5 Fgfrl1–/– embryos that died before birth only appeared after E14.5 when congenital heart defects were already present; therefore, placental phenotypes are

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**Fig. 4. Skeletal anomalies in Fgfrl1–/– embryos.** (A) Comparison between the whole skeletons of wild-type (+/+) and homozygous Fgfrl1 mutant (–/–) embryos at E18.5, with ossified areas in red and cartilage in blue, revealed a general hypoplasia of all skeletal elements in Fgfrl1 mutant embryos, including shortened axial and appendicular skeletons, malformation of the vertebrae (arrowhead), shortening of the limb girdles and a reduction in the size of the ribcage. (B) Ventral view of the skull, showing midfacial and mandibular hypoplasia and an anterior-shifted foramen magnum in Fgfrl1–/– mice. (C) Dorsal view of the skull, revealing a delay in suture closure between frontal and parietal bones in Fgfrl1–/– mice. The frontal and parietal bones in Fgfrl1–/– mice are also thin compared with those in wild-type littermates. Comparison between cross sections of wild-type (D) and homozygous mutant (E) shows enlargement of the brain ventricles and brain overgrowth (arrow) in Fgfrl1–/– mice. (F,G) Ossification of the cervical vertebrae (arrowhead) is delayed in homozygous mutants. (H,J) Fgfrl1–/– mice display a shortened sternum with abnormal ossification that is most prominent in the manubrium and the xiphoid process. The sternum was also bending inward in the mutant background, contributing to the reduced thoracic diameter. (I,K) Fgfrl1–/– mice show incomplete ossification of the hyoid bone and hypoplasia of permanent laryngeal cartilage elements. Abbreviations: bo, basioccipital; bs, basiphenoid; cc, cricoid cartilage; fm, foramen magnum; fr, frontal; hb, hyoid bone; ma, manubrium; pa, parietal; sp, sphenoid; tc, thyroid cartilage; tr, trachea; xp, xiphoid process. Bars, 1 mm.
likely to be secondary to other cardiovascular defects in the cause of prenatal death.

To further investigate the cause of prenatal lethality, we also searched for potential haematopoietic defects. Complete blood count analysis of \textit{Fgfrl1}\textsuperscript{−/−} E16.5 embryos revealed a decrease in red blood cell count (27%), haematocrit (23%) and haemoglobin (37%) (Fig. 7A-C). Although definitive haematopoiesis starts on E12 in foetal mouse liver, the erythroid differentiation process only reaches a steady state after E15.5 (Zhang et al., 2003). To characterise the anaemic state in \textit{Fgfrl1}\textsuperscript{−/−} embryos, we further assessed the maturation stage of foetal liver erythropoiesis by flow cytometry. Although no differences were observed in the absolute counts of foetal liver cells, or cells in the more primitive proerythroblast compartment (c-kit\textsuperscript{+}/Ter119\textsuperscript{−}, CD71\textsuperscript{+}/Ter119\textsuperscript{−}) (Fig. 7D and data not shown), the flow cytometric profile of the mature (Ter119\textsuperscript{+}) cells was affected in \textit{Fgfrl1}\textsuperscript{−/−} E14.5 embryos. An increase in basophilic erythroblasts (R3, 7%) and a decrease in the more mature erythroblasts (R4, 19%; R5, 35%) reflected a delay in the differentiation of erythroid progenitor cells. After two days, \textit{Fgfrl1}\textsuperscript{−/−} E16.5 foetal liver cells showed a normal pattern of erythroid differentiation (Fig. 7D), and at E18.5, peripheral blood displayed normal levels of red blood cells and haemoglobin (data not shown).

**DISCUSSION**

In this study we present an in-depth characterisation of \textit{Fgfrl1}\textsuperscript{−/−} mice that recapitulate multiple aspects of human WHS. These data support a wider role for \textit{Fgfrl1} in development and implicate \textit{FGFRL1} insufficiency in the human syndrome. Here, the mouse model does not perfectly recapitulate the human syndrome: in human patients, \textit{FGFRL1} haploinsufficiency within the context of a large deletion has been implicated in the craniofacial phenotype of WHS (Engbers et al., 2009), whereas, in the mouse mutants described in the present and previous studies (Baertschi et al., 2007), heterozygous \textit{Fgfrl1} null mutants have no discernible phenotype. Several explanations for this discrepancy can be forwarded. First, developmental processes often involve threshold effects that can be influenced by the genetic background: the literature is rife with examples of null mutations in mice that cause lethal defects on one inbred background, but which are relatively harmless on another (LeCouter et al., 1998; Becker et al., 2003; Teng et al., 2007). In this context, the lack of a phenotype in \textit{Fgfrl1}\textsuperscript{+/−} mice may recapitulate

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**Table 3. Distance between the proximal and distal growth plates of long bones at E18.5**

<table>
<thead>
<tr>
<th>Bone</th>
<th>Wt (n=11)</th>
<th>\textit{Fgfrl1}\textsuperscript{−/−} (n=11)</th>
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<tr>
<td>Humerus</td>
<td>2.36±0.11</td>
<td>1.74±0.17*</td>
</tr>
<tr>
<td>Ulna</td>
<td>2.72±0.08</td>
<td>2.18±0.25*</td>
</tr>
<tr>
<td>Femur</td>
<td>1.97±0.10</td>
<td>1.55±0.20*</td>
</tr>
<tr>
<td>Tibia</td>
<td>2.40±0.09</td>
<td>1.94±0.24*</td>
</tr>
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Long bones of \textit{Fgfrl1}\textsuperscript{−/−} mice were 19-26% shorter than in wild-type mice and this difference was statistically significant. Mann-Whitney U test, *\textit{P}<0.0001. Length in millimetres (±S.D.).

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**Fig. 5. Congenital heart defects in \textit{Fgfrl1}\textsuperscript{−/−} embryos.** At E14.5, a comparison between wild-type (A-C) and \textit{Fgfrl1}\textsuperscript{−/−} (D-F) hearts reveals ventricular septation defects [compare (A) with (D)], and thickening of the atrioventricular [compare (B) with (E)] and semilunar [compare (C) with (F)] valves (arrowheads) in \textit{Fgfrl1}\textsuperscript{−/−} embryos. (G-L) The \textit{Fgfrl1}\textsuperscript{−/−} mutant phenotypes persist at E18.5: ventricular septation defects [compare (G) with (J)], and thickening of the atrioventricular [compare (H) with (K)] and semilunar [compare (I) with (L)] valves (arrowheads). Bars, 0.5 mm.
those human genetic backgrounds where the deleterious effects of heterozygous Fgfrl1 mutations might be masked and go undetected.

Second, in a comparative analysis of WHSCRs, aligning mammalian genes that are orthologous to those at the human 4p16.3 locus (Fig. 8) reveals that, in rodents, this region has been broken up, separating Fgfrl1 and its neighbouring genes from the WHSCRs over rather distant locations (~75 Mb) on the same chromosome, whereas primates and ungulates maintain a high degree of conservation in chromosomal organisation. A mammalian phylogeny based on molecular data has shown that primates and rodents are more closely linked to each other than to ungulates, which probably represent an ancestral outgroup to the primate-rodent clade (Murphy et al., 2001). The separation of rodent Fgfrl1 from the WHSCRs may, therefore, be the result of a more recent event, perhaps involving an inversion. This complicates the interpretation of the large-scale mouse deletions, as described by Näf et al. (Näf et al., 2001), since the emerging model of human WHS predicts that many mutations in this region are implicated in the syndrome: deletion of any of at least three non-overlapping regions in 4p16.3 can be pathogenic for the craniofacial features of WHS (South et al., 2008). Thus, it is likely that in humans, heterozygous mutations in both FGFRL1 and a homologue of an undefined gene that is eliminated in WHS mouse models with large-scale deletions that do not target Fgfrl1 (Näf et al., 2001), collectively contribute to the craniofacial features of WHS.

Finally, differential regulation of human FGFRL1 and mouse Fgfrl1 gene expression may account for the mouse-human differences. In this scenario, the human FGFRL1 gene may be controlled by proximal regulatory elements that are within the WHSCRs or further towards the centromere; these elements lie distal to the rearranged mouse Fgfrl1 gene locus, changing the regulatory landscape, thereby exacerbating the effects of haploinsufficiency.

These considerations notwithstanding, Fgfrl1–/– mice recapitulate a remarkable number of malformations associated with WHS. Typical WHS skeletal phenotypes (Battaglia et al., 2001) in mutants that develop to term include a short stature, associated shortening of the pelvic girdle and appendicular skeleton, craniofacial dysgenesis, malformed vertebrae, delayed ossification of cervical vertebrae, abnormal sternal ossification and an associated reduction of the thoracic diameter. Laryngeal cartilage elements in Fgfrl1–/– mice are significantly reduced in size and provide an explanation for the problems in swallowing and speech development in WHS patients. The fully penetrant diaphragm phenotype of Fgfrl1–/– mutants might underlie the diaphragmatic hernia observed in some WHS patients (van Dooren et al., 2004).

Table 4. Congenital heart defects in Fgfr11−/− mice at different developmental stages

<table>
<thead>
<tr>
<th>Cardiac defects</th>
<th>E14.5</th>
<th>E16.5</th>
<th>E18.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membranous VSD</td>
<td>16</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Muscular VSD</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Thickened semilunar valves</td>
<td>16</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>Thickened atrophic valves</td>
<td>16</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>Thin myocardium</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Total number of Fgfr11−/− mice</td>
<td>16</td>
<td>7</td>
<td>11</td>
</tr>
</tbody>
</table>

VSD = ventricular septation defect.

Fig. 6. Comparison of placental development between wild-type (+/+) and homozygous Fgfrl1 mutant (−/−) embryos. (A,B) Histological analysis of placentas at E14.5 reveals no gross morphological differences between wild-type and Fgfrl1−/− embryos. (C) Placentas of severely affected Fgfrl1−/− embryos at E16.5 were pale compared with those of wild-type littermates. At E16.5, mildly affected Fgfrl1−/− embryos have a normal placenta (compare (D) with (E)), whereas the structure of the labyrinth in placentas of severely affected Fgfrl1−/− embryos is highly disorganised (F). Abbreviations: de, decidua; la, labyrinth; sp, spongiotrophoblast. Bars, 1 mm.
and, together with the abnormal laryngeal cartilage elements, contribute to WHS respiratory diseases. Fgfrl1−/− mice also display ventricular septation defects and abnormal thickening of both atrioventricular and semilunar valves, in line with common congenital heart malformations, such as pulmonary stenosis and aortic insufficiency, in WHS patients (Battaglia et al., 2001). Together with these cardiac defects, the surviving Fgfrl1−/− neonates displayed a combination of congenital malformations including a small rib cage and a fully penetrant diaphragm phenotype that, cumulatively, cause respiratory problems which led to perinatal lethality. By contrast, the Fgfr1−/− mice generated previously by Baertschi et al. (Baertschi et al., 2007) display the same diaphragm phenotype observed here, but these mice did not reveal any further defects in skeletal or cardiac development. The discrepancy is probably the result of differences in mutant gene construction: in the study by Baertschi et al., the first two exons were removed, potentially preserving partial function of the remaining encoded protein.

The skeletal phenotype of Fgfrl1−/− mice at E18.5 is similar to that of the achondroplasia-like dwarfism in transgenic mice that express a constitutively active mutant of MEK1 in chondrocytes, and phosphorylation of MEK1 in the growth plate chondrocytes is caused, at least in part, by signals originating from FGFR3 (Murakami et al., 2004). Interestingly, the FGFR3 locus, which is proximal to WHSC1 on the short arm of chromosome 4, has also been cited as a candidate gene contributing to the WHS phenotype (Bergemann et al., 2005). Different missense mutations of human FGFR3 underlie several skeletal dysplasias, including the most common form of human skeletal dysplasia, achondroplasia, which causes dwarfism (Rousseau et al., 1994; Shiang et al., 1994). In mice, Fgfr3 is expressed in the proliferating chondrocytes of the growth plate, and gain-of-function mutations, identical to those found in

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**Fig. 7. Foetal anaemia in Fgfr1−/− embryos.** (A-C) The peripheral blood in E16.5 Fgfr1−/− embryos shows a 27% decrease in red blood cell count (A), a 37% decrease in the haemoglobin level (B) and 23% less haematocrit (C) compared with wild-type littermates. Data are presented as mean±s.e.m. (***P<0.01). (D) Dot plots of the typical CD71 and Ter119 staining pattern on 10,000 viable cells that were dispersed from wild-type (WT) and Fgfr1−/− (KO) whole foetal livers at E14.5 and E16.5. The axes indicate the relative logarithmic fluorescence units for Ter119-PE-Cy7 (x-axis) and CD71-PE (y-axis). The cells in regions R1 to R5 represent erythroblasts at different developmental stages (R1 contains the least, and R5 the most, differentiated cells) and are defined by their characteristic CD71 and Ter119 staining pattern. These regions can be classified as follows: primitive progenitor cells and proerythroblasts (R1), proerythroblasts and early basophilic erythroblasts (R2), early and late basophilic erythroblasts (R3), chromatophilic and orthochromatophilic erythroblasts (R4), and late orthochromatophilic erythroblasts and reticulocytes (R5) (Zhang et al., 2003). The numbers shown in the boxed areas indicate the frequency of cells in each region, as a percentage of total liver cells. Asterisks indicate the regions (R3, R4, R5) with significantly different values between Fgfr1−/− and wild-type embryos at E14.5 (P<0.01).

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**Fig. 8. Comparison between WHSCRs in different mammalian species.** Alignment between the human and orangutan (primates), mouse and rat (rodents), and horse and cow (ungulates) orthologous genes, which in humans are located at 4p16.3, reveals a high degree of conservation in chromosomal organisation between primates and ungulates, but shows that rodents have a very different arrangement. The chromosomal region that encompasses a part of 4p16.3 in humans has been broken up in rodents, separating Fgfr1 from the WHSCRs over rather distant locations on the same chromosome (~75 Mb in the mouse and ~81 Mb in the rat). (Sequences have been derived from the Ensembl Genome Browser.)
humans, also cause achondroplasia, mimicking the human dwarf phenotype (Naski et al., 1998). By contrast, Fgfrl1 is specifically expressed in the stem-like cells in the resting zone (Lazarus et al., 2007), which provide the proliferating chondrocytes to the growth plate. The long bones in Fgfrl1–/– mice are significantly shorter than in wild-type littermates, whereas Fgfr3-deficient mice show bone overgrowth (Colvin et al., 1996). Together, Fgfrl1 and Fgfr3 might antagonize each other to control chondrocyte proliferation and it will be interesting to determine whether Fgfrl1 and Fgfr3 genetically interact to coordinate bone growth.

Our analysis of the early stages of development revealed that Fgfrl1 is expressed in the brain, the cranial placodes, the somites and the pharyngeal arches, suggesting that the Fgfrl1 expression pattern is conserved between mice, Xenopus (Hayashi et al., 2004) and zebrafish (Hall et al., 2006). Zebrafish fgfrl1a is also expressed in the arch 1- and 2-associated neural crest, and its depletion results in a craniofacial phenotype, including malformations of the lower jaw and developmental inhibition of the cartilage that is formed by the branchial arches that give rise to the gills (Hall et al., 2006). In mice, the neural crest cells of the first pharyngeal arch form the jawbones and the bones of the face, whereas more posterior pharyngeal arches participate in the development of the hyoid bone and the permanent laryngeal cartilage elements (Noden and Trainor, 2005).

Congenital heart defects at E14.5 probably contribute to prenatal lethality among Fgfrl1–/– mice. Expression of Fgfrl1 in the endocardial cushions of the developing outflow tract might implicate neural crest-derived tissue in these defects. A role for FGF signalling in outflow tract development is illustrated by the persistent truncus arteriosus observed in Fgfr8 mutants (Frank et al., 2002). In addition, congenital heart diseases such as DiGeorge syndrome display persistent truncus arteriosus owing to a 22q11 deletion that results in the loss of TBX1, which in mice genetically interacts with Fgfr8 to coordinate cardiac neural crest cells for outflow tract remodelling (Stoller and Epstein, 2005). Our analysis indicates that Fgfrl1 is not involved in this signalling network, since persistent truncus arteriosus was not present in Fgfrl1–/– mice, suggesting that the cardiac neural crest cells that populate the endocardial cushions of the outflow tract were unaffected. Cardiac neural crest cells and endocardial cells both populate the outflow tract cushions, but cardiac valves and part of the ventricular septum are derived from the endocardial cell lineage without a contribution from neural crest cells (de Lange et al., 2004). The specific expression of Fgfrl1 in the outflow tract and atrioventricular endocardial cushions, in combination with the consistent valve phenotype and ventricular septation defect in Fgfrl1–/– mice, support a role for Fgfrl1 in coordinating cells that are derived from the endocardial lineage to form the valve complexes and to contribute to the ventricular septum.

Further investigation into the potential causes of prenatal lethality led us to focus on the haematopoietic system. At E16.5, Fgfrl1–/– mice display anaemia, with the 37% decrease in haemoglobin evidently limiting the oxygen supply to embryonic tissues. The cause for this defect is likely to be the delayed maturation of erythroid progenitors at E14.5. This was a transient problem in Fgfrl1+/– mice, because by E16.5 there was no abnormal pattern in liver cell distribution and, at E18.5, normal levels of red blood cells, haematocrit and haemoglobin were observed in the peripheral blood. Nevertheless, persistent haematological disorders have been observed in patients with WHS (Sharathkumar et al., 2003) and our results further support the importance of evaluating children with WHS for haematopoietic dysfunction. Together with cardiac defects, foetal anaemia was present in all Fgfrl1–/– mice at E14.5, with no distinction between the potential severely and mildly affected mutants. The varying severity of the combined congenital heart defects and transient foetal anaemia in Fgfrl1–/– mice probably represents a stochastic process that underlies the observed prenatal lethality in a portion of mutant embryos.

FGF signalling plays a crucial role in a wide range of developmental processes, including cartilage formation in the vertebrate head (Walshe and Mason, 2003), suture closure (Connerney et al., 2008), bone growth (Kronenberg, 2003) and cardiac valve formation (Sugi et al., 2003). Although the molecular function of FGFR1 in these processes remains unknown, Trueb et al. (Trube et al., 2003) have suggested that FGFR1 might act as a decoy receptor to coordinate the distribution of free FGF ligand. This possibility is supported by the function of the FGFR-related gene nou-darake in planarian worms, which is similar to Fgfrl1 and inhibits FGF signalling in Xenopus eggs (Cebria et al., 2002). Fluorescence resonance energy transfer (FRET) studies demonstrate that FGFR1 proteins form constitutive homodimers at the cell surface, which promote cell adhesion properties in a similar way to the nectins (Rieckmann et al., 2008). This is consistent with the phenotypic alterations observed in Fgfrl1–/– mice, where the absence of Fgfrl1 may adversely affect epithelial-to-mesenchymal transition of endocardial cells in the outflow tract cushions or the migration of neural crest cells in the pharyngeal arches. Our study thus provides an animal model to investigate mechanistic aspects of mammalian FGFR1 function that could shed light on the complex aetiology of human WHS disease.

METHODS
Gene targeting
We used homologous recombination to target Fgfrl1 to produce the Fgfrl1+/- allele. The targeting construct was generated by inserting part of the Fgfrl1 locus of a C57BL/6J bacterial artificial chromosome (BAC) clone (BACPAC Resources, CHORI), including a 7.0 kb fragment of intron 2 (5' arm), exons 3 to 7 (3.1 kb), and a 4.2 kb fragment from downstream of the last exon (3' arm), into a pBluescript vector by RecE-RecT recombination (Anstrand et al., 1999). Generation ofloxP sites was also performed by using RecE-RecT recombination. A loxP-flanked PGK neo' cassette was inserted 5' of exon 3, used for selection and later removed by electroporating positive clones with a Cre-recombinease-expressing plasmid, leaving a single 5' loxP site. The FRT-flanked PGK neo' cassette and an additional loxP site were inserted 3' of the coding sequence. The final Fgfrl1 targeting construct was confirmed by sequencing analysis.

The Fgfrl1 targeting vector was linearised with NotI, transfected by electroporation into 129/Sv embryonic stem cells and neomycin-resistant colonies were then screened by Southern blot.
hybridisation using 5’ and 3’ external probes with SpeI-digested genomic DNA. Additional restriction digests with BamHI and EcoRI confirmed the targeting event. Two properly targeted ES cell clones were injected into C57BL/6J blastocysts to generate chimeric mice. Chimeric males were mated with C57BL/6J females and the agouti coat colour, Southern blot and PCR analysis confirmed germine transmission. Heterozygous mice grew normally, and the exons 3 to 7 and the PGK neo+ cassette were removed in vivo with a CMV-Cre deleter strain (Schwenk et al., 1995). The phenotypes of the two independent lines were similar. Newborn Fgfrl1–/+ mice were occasionally found half-eaten, which explains why, in many litters, we did not find any Fgfrl1–/– mice (see Table 1). Mice with the Fgfrl1lox allele and the Fgfrl1+/– allele were genotyped by PCR with the primers P1 (5’-CCAGAGTTGCGGTTCCAG-3‘), P2 (5’-GCTGCTGGATACACATG-3’) and P3 (5’-GGCATGGCTGCAGTC-3’), using the following parameters: 94°C for 5 minutes, followed by 35 cycles of 94°C for 1 minute, 56°C for 30 seconds and 72°C for 30 seconds. All experiments were performed on mice with a ≥F5 C57BL/6J background and n≥10 unless indicated otherwise. All mouse procedures were approved by the European Molecular Biology Laboratory ethical committee (Monterotondo, Italy) and were in accordance with national and European regulations.

In situ hybridisation
Full-length Fgfrl1 cDNA in a pCMV-SPORT6 vector (imaGenes) was digested with Xhol or EcoRI to generate sense and antisense probes, respectively, using a DIG RNA labelling kit (Roche). Embryos were fixed in phosphate buffered saline (PBS)/4%/paraformaldehyde (PFA) and stored in methanol. In situ hybridization was performed as described previously (te Welscher et al., 2002). Paraffin sections were counterstained for 5 minutes with Nuclear Red (Vector) and washed in water.

Histological analysis
Embryonic tissues were dissected and fixed in PBS/4%/PFA, gradually dehydrated in ethanol and embedded in paraffin. Tissue sections (8 μm) were analysed after Trichrome staining (Sigma-Aldrich).

Quantitative real time (RT)-PCR
Total RNA was prepared from E12.5 embryos using TRIzol (Invitrogen), DNase (Promega)-treated and cleaned by column purification (Qiagen). After quantification by spectroscopy, 2 μg of RNA was used for cDNA synthesis (SuperScript first-strand synthesis system for RT-PCR, Invitrogen). Inventoried FAM TaqMan probes (Applied Biosystems) were used for the relative quantification of the mRNA levels of Fgfrl1 (Mm01198875_g1), Fgfr3 (Mm01211080_m1), Whsc1 (Mm01211108_m1) and Whsc2 (Mm01217228_m1). Taqman VIC glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probes (4352339E) were used for normalisation.

Skeletal staining
E18.5 embryos were skinned and eviscerated before fixation in 95% ethanol overnight. For cartilage staining, embryos were placed overnight in Alcian Blue solution (0.1 g Alcian Blue 8GX in 160 ml of 95% ethanol and 40 ml of glacial acetic acid) and transferred subsequently to 95% ethanol for 3 hours. For bone staining, embryos were cleared in 2% KOH solution for 24 hours and placed overnight in Alizarin Red solution (0.03 g Alizarin Red in 1% KOH solution). Clearing of the embryos was completed in 1% KOH with 20% glycerol for 2 days, and the embryos were then stored in a mixture of ethanol/glycerol (1:1).

Flow cytometric analysis
Single cell suspensions prepared from E14.5 (Fgfrl1+/–, n=13; wild type, n=7) and E16.5 (Fgfrl1–/–, n=7; wild type, n=19) foetal livers were filtered (70 μm), washed by centrifugation and resuspended in PBS containing 1% foetal calf serum. Cells were incubated with Fc block (CD16/CD32) for 15 minutes on ice and stained with antibodies for 15 minutes on ice. The antibodies allophycocyanin-conjugated anti-c-Kit, phycoerythrin-conjugated anti-CD71 and PE-Cy7-conjugated Ter119 were obtained from BD Biosciences. Analysis was performed in a two-laser, standard-configuration fluorescence-activated cell sorter (FACS) Canto (BD Biosciences). Gating of the different erythroid populations was performed as described previously (Zhang et al., 2003). Compensation was performed automatically using FACSDiva software 6.0. Data analysis was performed using FlowJo software 8.6 (TreeStar). An
equivalent number of events were analysed and displayed graphically in each set of experiments.

Blood analysis
Analysis of peripheral blood was performed with the Drew Scientific Hemavet 950.

Bone measurements
Digital images of long bones were analysed using the MetaMorph software (Version 7.1.2; Molecular Devices).

Statistical analysis
Statistical significance was determined with the unpaired two-tailed Student’s t-test or with the Mann-Whitney U-test, as appropriate for small sample sizes.

URL
The Ensembl Genome Browser is available at http://www.ensembl.org/.

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COMPETING INTERESTS
The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS
C.C., histological analysis and contributions to experimental design; D.B.-C., flow cytometric analysis; E.S., genotyping; P.K., RT-PCR analysis; N.R., contributions to experimental design and writing of the manuscript; P.t.W., experimental design, cytometric analysis; E.S., genotyping; P.K., RT-PCR analysis; N.R., contributions to experimental design and writing of the manuscript with input from C.C. and D.B.-C.

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