A *Grhl2*-dependent gene network controls trophoblast branching morphogenesis

Katharina Walentin1,2, Christian Hinze1,2, Max Werth1,2,3, Nadine Haase2, Saaket Varma4, Robert Morell5, Annekatrin Aue1,2, Elisabeth Pötschke1, David Warburton6, Andong Qiu2, Jonathan Barasch3, Bettina Pürß1, Christoph Dieterich4, Elena Popova1, Michael Bader1, Ralf Dechend2, Anne Cathrine Staff7, Zeliha Yesim Yurtdas1,8,9, Ergin Kilic10 and Kai M. Schmidt-Ott1,2,11,*

INTRODUCTION

Healthy placental development is essential for reproductive success; failure of the feto-maternal interface results in pre-eclampsia and intrauterine growth retardation. We found that grainyhead-like 2 (*Grhl2*), a CP2-type transcription factor, is highly expressed in chorionic trophoblast cells, including basal chorionic trophoblast (BCT) cells located at the chorioallantoic interface in murine placentas. Placentas from *Grhl2*-deficient mouse embryos displayed defects in BCT cell polarity and basement membrane integrity at the chorioallantoic interface, as well as a severe disruption of labyrinth branching morphogenesis. Selective *Grhl2* inactivation only in epiblast-derived cells rescued all placental defects but phenocopied intraembryonic defects observed in global *Grhl2* deficiency, implying the importance of *Grhl2* activity in trophoderm-derived cells. ChIP-seq identified 5282 *Grhl2* binding sites in placental tissue. By integrating these data with placental gene expression profiles, we identified direct and indirect *Grhl2* targets and found a marked enrichment of *Grhl2* binding adjacent to genes downregulated in *Grhl2*-deficient placentas, which encoded known regulators of placental development and epithelial morphogenesis. These genes included that encoding the serine protease inhibitor Kunitz type 1 (*Spint1*), which regulates BCT cell integrity and labyrinth formation. In human placenta, we found that human orthologs of murine *Grhl2* and its targets define presumptive branch points. *Grhl2*-coordinated gene network controls trophoblast branching morphogenesis, thereby facilitating development of the site of feto-maternal exchange. This might have implications for syndromes related to placental dysfunction.

KEY WORDS: Placenta defects, Epithelial differentiation, Epithelial morphogenesis, *Spint1*, Basement membrane defects

RESEARCH ARTICLE

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BCT cells of mice (Hallikas et al., 2006; Szabo et al., 2007). Comparative genomics has shown that orthologs of over 80% of the genes known to be required for proper placental development in mice are also expressed in human placenta (Cox et al., 2009). The precise molecular programs driving branching morphogenesis, invasion and trophoblast differentiation remain incompletely understood.

Grainyhead-like transcription factors regulate the development of epithelial cell types in several species. They are implicated in epithelial morphogenesis, barrier formation and wound healing processes (Bray and Kafatos, 1991; Mace et al., 2005; Tao et al., 2005; Narasimha et al., 2008; Yu et al., 2009; Han et al., 2011; Gao et al., 2013). Grhl2 is one of three mouse homologs of Drosophila Grainyhead (Wilanowski et al., 2002) and is expressed in diverse embryonic epithelial tissues during development (Wilanowski et al., 2002; Auden et al., 2006). Grhl2 and its paralog Grhl3 play essential roles in neural tube closure in mice (Rifat et al., 2010; Werth et al., 2010; Brouns et al., 2011; Pyrgaki et al., 2011). All three members regulate the expression of epithelial junctional genes (Yu et al., 2006; Wilanowski et al., 2008; Werth et al., 2010; Pyrgaki et al., 2011; Senga et al., 2012; Varma et al., 2012). We now report that Grhl2 controls a target gene set in placental trophoblasts and is thereby crucial to placental morphogenesis.

RESULTS

Grhl2 ablation in mice perturbs placental labyrinth formation

We previously reported the generation of two mouse Grhl2 null alleles: Grhl2<sup>lacZ1</sup> and Grhl2<sup>lacZ4</sup> (Werth et al., 2010). Whereas heterozygous Grhl2<sup>+/+</sup> and Grhl2<sup>+/−</sup> mice appeared normal, no live homozygous Grhl2<sup>+/−</sup> and Grhl2<sup>−/−</sup> mice were recovered postnatally from heterozygous intercrosses (supplementary material Table S1). Until E11.5, the genotype distribution showed Mendelian ratios between Grhl2<sup>−/−</sup>, Grhl2<sup>+/−</sup> and Grhl2<sup>+/+</sup> mutants (collectively referred to as Grhl2<sup>−/−</sup>-). Analysis of embryos from timed heterozygous matings revealed that neural tube defects became apparent at E9.5 and IUGR at E10.5 in Grhl2<sup>−/−</sup> mutants. By E11.5, Grhl2<sup>−/−</sup> embryos were still present but had no visible heartbeat and displayed evidence of advanced tissue decay. We found no Grhl2<sup>−/−</sup> mutants after E11.5, indicating that Grhl2 is crucial for embryonic development and survival past this stage.

To test the possibility that placenta defects contribute to this phenotype, we first analyzed GRHL2 expression by immunohistochemistry, which revealed robust levels in trophoblast cells from E7.5 to E16.5 (Fig. 1). We found high expression in the chorion at E7.5 and E8.0 (Fig. 1A-D). At E9.0, GRHL2 expression in the chorion became restricted to BCT cells (Fig. 1E,F). Moreover, GRHL2 was expressed in primary and secondary trophoblast giant cells (TGCs) (Fig. 1A,G-I) and in sinusoidal TGCs (S-TGCs) (Fig. 1H-J, arrows) of the chorion-derived labyrinth. By contrast, GRHL2 expression was low in the ectoplacental cone (Fig. 1A-D), but became more prominent as this structure developed into the spongiotrophoblast (Fig. 1G,I). We found no GRHL2 expression in the extraembryonic and allantoic mesoderm or in the maternal decidua (for an overview of the GRHL2 expression domain see Fig. 1K). No GRHL2 staining was present in Grhl2<sup>−/−</sup> placentas (supplementary material Fig. S1).

Histological analysis of mutant placentas in comparison with littermate controls revealed no abnormalities at E8.0 (supplementary material Fig. S2A,B). Occlusion of the ectoplacental cavity and chorioallantoic attachment occurred normally, resulting in chorioc plates in contact with the ectoplacental cone and allantois at E9.5 (Fig. 2A,B). In control placentas at E9.5, the initially flat chorion began to undergo branching morphogenesis to form the labyrinth, and fetal blood vessels from the allantois started extending into the developing villi (Fig. 2A). By contrast, Grhl2<sup>−/−</sup> mutants showed a marked disruption of chorioc branching, resulting in flat and compact chorioc plates with scant fetal blood vessel ingrowth (Fig. 2B,D) and in reduced thickness of the labyrinth at E10.5 (Fig. 2C,D, arrows). Labeling of fetal blood vessels and maternal blood sinuses in H&E-stained E10.5 sections based on the presence of nucleated (fetal) or non-nucleated (maternal) erythrocytes revealed a complex network of fetal and maternal blood spaces in controls, whereas Grhl2<sup>−/−</sup> placentas exhibited a marked simplification (Fig. 2E,F). Fetal vasculature, as labeled by PECAM1 staining, largely remained in the allantoic...
compartment and failed to permeate into the pan-cytokeratin-labeled trophoblast layer (Fig. 2G,H; supplementary material Fig. S2C,D). GRHL2 was not expressed in fetal blood vessels themselves (Fig. 1), suggesting that the reduced fetal vascularization was secondary to defective trophoblast branching. Co-immunostaining of the labyrinth markers transferrin receptor and ferroportin (SLC40A1 – Mouse Genome Informatics), which label STB layer I and II, respectively, demonstrated a progressively complex and expanding STB compartment in controls at E10.0 and E11.5, but markedly defective development in Grhl2−/− placentas, with scant evidence of STB differentiation in tip regions (Fig. 2I-L; supplementary material Fig. S2E,F). Although trophoblast branching morphogenesis was initiated, it failed to progress appropriately in mutants (Fig. 2J,L; supplementary material Fig. S2F). Moreover, STB layer II appeared thickened at differentiation sites, as indicated by ferroportin staining (supplementary material Fig. S2G,H). Together, these findings suggest a central role for Grhl2 in regulating labyrinth trophoblast morphogenesis and differentiation.

We next analyzed trophoblast-specific differentiation markers to determine the presence and quantity of different cell types. To label branch points in the chorionic layer, we stained for Gcm1 mRNA. As previously reported, control placentas showed Gcm1 staining in clusters at prospective branching initiation sites at E9.5 and at branch points in the labyrinth at E11.5 (supplementary material Fig. S3A-D) (Anson-Cartwright et al., 2000). In E9.5 Grhl2−/− placentas, Gcm1 was expressed at the sites of branch point initiation (supplementary material Fig. S3B). Consistently, trophoblast branching was initiated in mutants, indicating that the disturbed trophoblast branching was not related to defective Gcm1 expression. However, mutants contained markedly reduced numbers of Gcm1-positive branching initiation sites (supplementary material Fig. S3B,D), indicating a quantitative defect in branch point formation. The spongiotrophoblast marker Tpbpa was expressed in mutants (supplementary material Fig. S3E,F). We monitored the development and differentiation of TGC types by staining for specific markers Prl3d1, Prl3b1, Prl2c2 and Hand1 (supplementary material Fig. S3G-N), indicating that several hallmarks of TGC differentiation were unaltered in E9.5 Grhl2−/− placentas.

Since these data did not identify substantial defects outside the chorionic trophoblast compartment, we next focused on BCT cells,
which express high levels of GRHL2 and are known to drive chorionic branching. During placentaion, BCT cells were initially cuboid and became progressively flattened after allantoic attachment. Labeling with the trophoblast marker TCFAP2C confirmed the ordered appearance of BCT cells in wild-type placentas, but revealed disorganized and multilayered BCT cells in Grhl2 mutants (Fig. 2M, N). To analyze cell polarity in the BCT cell layer, we stained for integrin alpha 6 (ITGA6), a marker of the basolateral membrane domain. Whereas ITGA6 showed a strictly basolateral pattern in control BCT cells, its positioning appeared randomized in Grhl2−/− BCT cells, with cuboid cells that failed to flatten and branch (Fig. 2O, P), indicating a disruption of apicobasal polarization and of cell orientation in mutant BCT cells. Analysis of cell proliferation in the BCT layer via Ki-67 (MKI67) immunofluorescence showed no differences between controls and mutants at E9.5 (see supplementary material Fig. S4F-M). Hence, the defects observed in Grhl2−/− mice, we employed ChIP-sequencing (ChIP-seq) on mouse genomic analyses.

Identification of placental GRHL2 target genes by integrated genomic analyses

To understand the molecular basis of placenta defects in Grhl2−/− mice, we employed ChIP-sequencing (ChIP-seq) on mouse placenta tissue using a GRHL2 antibody that we had previously shown to be specific (Werth et al., 2010). We identified 5282 peaks (P<10−5) of GRHL2 binding across the mouse genome using MACS (Zhang et al., 2008) (Fig. 4 and Fig. 5A). GRHL2 ChIP peaks were clustered around transcriptional units, with the majority located in upstream regulatory regions and introns (Fig. 4A). De novo motif discovery within the top 10% of the GRHL2-associated DNA regions (528 peaks) using MEME (Bailey and Elkan, 1994) yielded a sequence (Fig. 4B) identical to previously identified grainyhead binding motifs (Gao et al., 2013). A comparison with known vertebrate transcription factor binding sites using TOMTOM (Gupta et al., 2007) revealed high similarity.

**Epiblast-specific Grhl2 deletion rescues placenta defects**

Loss of intraplacental GRHL2 could contribute to the profound embryonic phenotype of Grhl2−/− mice and might explain lethality after E10.5. However, the severe embryonic alterations in Grhl2−/− mice might also aggravate the placenta defects. Thus, we generated a conditional allele of Grhl2 (Grhl2flfox) allowing tissue-specific Cre-dependent Grhl2 ablation (supplementary material Fig. S5A). Sox2Cre mice enable Cre-mediated recombination selectively in the epiblast-derived embryo and epiblast-derived extraembryonic membranes, including amnion, yolk sac mesoderm and allantois, as shown in previous studies at E7.5 (Hayashi et al., 2002). We confirmed the absence of Sox2Cre-mediated recombination in E9.5 trophectoderm-derived trophoblasts by β-galactosidase staining in Sox2Cre;R26R-lacZ placenta sections, which revealed recombination only in the allantoic mesoderm (supplementary material Fig. S5B). We then crossed male Sox2Cre;Grhl2flfox/flfox mice with female Grhl2flfox/flfox mice to generate Sox2Cre;Grhl2−/− embryos. These embryos were present at Mendelian ratios up to E11.5. Their phenotype was indistinguishable from that of Grhl2−/− embryos (Fig. 3A-C; supplementary material Fig. SSC-F) and included split palate malformations, exencephaly, as well as anterior and lumbosacral spina bifida. Immunofluorescence staining confirmed the complete absence of GRHL2 protein in all extraembryonic tissues of Sox2Cre;Grhl2−/− mice (Fig. 3D-G). H&E-stained E9.5 to E11.5 placenta sections of Sox2Cre;Grhl2−/− embryos revealed that, in contrast to Grhl2−/− mice, trophoblast branching morphogenesis plus permeation and branching of fetal blood vessels occurred normally. The complex network of fetal blood vessels and maternal blood sinususes developed normally (Fig. 3H-P). Despite the rescue of placental defects, we only recovered Sox2Cre; Grhl2−/− embryos up to E11.5, with evidence of IUGR and tissue decay similar to Grhl2−/− mice. These data indicate that Grhl2 in epiblast-derived tissues accounted for intraembryonic defects, but not for the placenta defect, implying a crucial role for Grhl2 in trophectoderm-derived trophoblast lineages.

**Identification of placental GRHL2 target genes by integrated genomic analyses**

To understand the molecular basis of placenta defects in Grhl2−/− mice, we employed ChIP-sequencing (ChIP-seq) on mouse placenta sections, which revealed a defective interaction of fetal and maternal blood in E10.5 Grhl2−/− placentas (M), but not in controls (K). (K-M) Blood spaces with fetal nucleated erythrocytes and maternal non-nucleated erythrocytes are labeled in blue and red, respectively, revealing a defective interaction of fetal and maternal blood in E10.5 Grhl2−/− placentas (L), but not in controls (K). (K-M) Blood spaces with fetal nucleated erythrocytes and maternal non-nucleated erythrocytes are labeled in blue and red, respectively, revealing a defective interaction of fetal and maternal blood in E10.5 Grhl2−/− placentas (L), but not in controls (K).
to the motif recognized by transcription factor CP2-like 1 (TFCP2L1; $P<0.005$), another member of the CP2 family (Chen et al., 2008). GRHL2 binding sites detected in kidney epithelial cells (our unpublished data) compared with our placental GRHL2 peaks showed an overlap of 41%. Moreover, 3611 out of the 5282 GRHL2 ChIP peaks determined in placenta tissue mapped to syntenic regions of the human genome, of which 592 (16.4%) overlapped with GRHL2 binding sites recently identified in lung epithelial cells (Gao et al., 2013).

Moreover, we used microarrays to compare gene expression in Grhl2−/− versus control E7.5 and E9.5 placentas (Fig. 4C). The intersection of the top 150 genes downregulated in Grhl2−/− placentas at E7.5 and E9.5 yielded 33 genes (supplementary material Table S2). Interestingly, the previously identified embryonic Grhl2 target genes Cldn4 and E-cadherin (Cdh1) (Werth et al., 2010) were absent from this gene list. This points to a marked tissue specificity of Grhl2 functions and suggests that the target gene set of Grhl2 in placenta differs from that in other tissues. Consistently, CLDN4 protein expression analysis by immunofluorescence staining showed high CLDN4 levels in BCT cells compared with the overlying chorionic trophoblasts in controls, and we found no profound effect of Grhl2 deficiency on placental Cldn4 expression by in situ hybridization as well as immunostaining at E9.5 and E11.5 (supplementary material Fig. S6). As Cldn4 expression was maintained in BCT cells deficient for Grhl2, this suggests that alternative pathways maintain Cldn4 expression in these cells.

In contrast to the downregulated genes, the intersection of the top 150 genes upregulated in mutants at E7.5 and E9.5 revealed no overlap. In addition, genes downregulated in mutant placentas revealed a marked overrepresentation of GRHL2 peak occurrence in the vicinity of genes downregulated in Grhl2−/− placentas (linear interpolated density plot over 500 genes). The heat maps at the bottom visualize the intersection of the top 150 fold-ranked genes for E9.5 and E7.5 separately. Blue bars indicate the presence of a GRHL2 ChIP peak near the respective gene. (D) Quantifying GRHL2 peak occurrence in the top 150 downregulated (blue) and upregulated (red) genes revealed a marked enrichment in genes downregulated in mutants ($P$-values calculated using Fisher’s exact test).

Fig. 4. Integration of ChIP-seq and microarray data identifies a putative placental GRHL2 target gene set. (A) Annotation of GRHL2 ChIP peaks obtained with E17.5 placenta tissue. The majority of GRHL2 peaks were clustered around transcriptional units and in upstream regulatory regions and introns (outer circle). The inner circle represents the annotation of an equal number of random peaks. TSS, transcription start site; TTS, transcription termination site; UTR, untranslated region. (B) De novo motif discovery from the top 10% GRHL2 ChIP peaks identified a sequence that is identical to previously published grainyhead binding motifs. $P$-value as provided by MEME. (C) Combination of GRHL2 ChIP peak and gene expression data analysis visualized by heat maps. In the center panel, heat maps are shown for all genes according to Illumina microarray analysis ordered by fold rank in E9.5 wild-type control (wt) versus Grhl2−/− (ko) placenta. Each numbered row represents one biological replicate. The dashed lines mark the top 150 genes. Genes at E7.5 are shown beneath in the same order. The GRHL2 peak density plot (top panel) corresponding to the genes shown in the heat maps beneath visualizes an increased frequency of GRHL2 ChIP peak occurrence in the vicinity of genes downregulated in Grhl2−/− placentas (linear interpolated density plot over 500 genes). The heat maps at the bottom visualize the intersection of the top 150 fold-ranked genes for E9.5 and E7.5 separately. Blue bars indicate the presence of a GRHL2 ChIP peak near the respective gene. (D) Quantifying GRHL2 peak occurrence in the top 150 downregulated (blue) and upregulated (red) genes revealed a marked enrichment in genes downregulated in mutants ($P$-values calculated using Fisher’s exact test).
within the top 150 genes downregulated in E7.5 and E9.5 Grhl2−/− placentas. Both analyses revealed a significant overrepresentation of genes involved in epithelial cell differentiation, epithelial morphogenesis and placenta development (supplementary material Table S3).

To determine a set of putative GRHL2 targets, we identified genes that were among the top 150 downregulated in mutant placentas at E7.5 and E9.5 and displayed a GRHL2 binding peak within a ±2 kb region around the gene. This yielded a module of 13 putative placental GRHL2 core targets (Fig. 4C,E, Fig. 5A, Table 1). ChIP-PCR using E9.5 placenta tissue confirmed specific enrichment at the putative GRHL2 binding areas of all 13 target genes when compared with the IgG control, and thus confirmed GRHL2 binding peaks identified by ChIP-seq (Fig. 5B). Validation of expression differences by quantitative PCR confirmed a significant downregulation of

Fig. 5. Identification and validation of putative placental GRHL2-dependent target genes.

(A) Depicted are all genes of the putative core target gene set (see Table 1) with a GRHL2 binding peak and showing significant downregulation in Grhl2−/− placentas as compared with wild-type controls based on microarray analysis (P<0.05). Peak occurrence and corresponding binding motifs are depicted at genomic loci of all target genes identified by GRHL2 ChIP-seq using E17.5 placenta tissue. ChIP peaks (blue) are visualized using QuEST (Valouev et al., 2008). (B) Validation of GRHL2 ChIP-seq peaks by ChIP with subsequent quantitative PCR analysis performed on E9.5 placenta tissue with normalization for total input. IgG was used as antibody control. Bars represent the median and error bars show the 25th and 75th percentile; n=3 biological replicates. *P<0.01, **P<0.01 versus IgG (Mann–Whitney U test). (C) Real-time RT-PCR confirmed significant downregulation of all GRHL2 targets in E9.5 Grhl2−/− placentas compared with wild-type controls identified by microarray analysis. β-actin was used as internal standard. Error bars represent mean±s.d.; n=6 biological replicates. *P<0.005, **P<0.01, ***P<0.001 versus wild-type control (Mann–Whitney U test).
Table 1. Core set of GRHL2-dependent genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold change microarray</th>
<th>Peak location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kcnk1</td>
<td>1.3</td>
<td>Intron 1</td>
</tr>
<tr>
<td>Lad1</td>
<td>1.5</td>
<td>TSS, intron 4</td>
</tr>
<tr>
<td>Ldoc1</td>
<td>1.9</td>
<td>3′ UTR</td>
</tr>
<tr>
<td>Plac1</td>
<td>1.9</td>
<td>−1134 bp</td>
</tr>
<tr>
<td>Prom2</td>
<td>3.2</td>
<td>TSS</td>
</tr>
<tr>
<td>Rab15</td>
<td>1.9</td>
<td>Intron 1</td>
</tr>
<tr>
<td>Smpd3b</td>
<td>1.5</td>
<td>TSS</td>
</tr>
<tr>
<td>Spint1</td>
<td>1.3</td>
<td>+63 bp</td>
</tr>
<tr>
<td>Tex19.1</td>
<td>2.0</td>
<td>−736 bp</td>
</tr>
<tr>
<td>Tgap2a</td>
<td>1.9</td>
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<td>Tmem54</td>
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</tr>
<tr>
<td>Tnk1</td>
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<td>Intron 6</td>
</tr>
<tr>
<td>1600014K23Rik</td>
<td>1.4</td>
<td>1.9</td>
</tr>
</tbody>
</table>

Intersection of the top 150 fold-ranked downregulated genes (wild-type control versus Grhl2−/−) at E7.5 and E9.5 with additional GRHL2 peak occurrence yielded a set of 13 potential GRHL2 core targets. Peak location (bp) is relative to gene body.

all 13 genes in E9.5 mutant placentas (Fig. 5C). A literature search revealed that homozygous knockouts for Spint1 (Tanaka et al., 2005), Plac1 (Jackman et al., 2012), Tex19.1 (Tarabay et al., 2013), Kcnk1 (Millar et al., 2006), Tgap2a (Schorle et al., 1996; Zhang et al., 1996) and Prom2 (Tang et al., 2010) had been phenotypically characterized previously, with three, namely Spint1, Plac1 and Tex19.1, displaying a placenta phenotype. Whereas Plac1−/− and Tex19.1−/− mice exhibit milder placenta defects that manifest in late pregnancy (Jackman et al., 2012; Tarabay et al., 2013), Spint1−/− mice have been reported to display an early placenta defect with lethality at E10.5 (Tanaka et al., 2005; Fan et al., 2007; Szabo et al., 2007), similar to the phenotype we found in Grhl2−/− mice.

**Grhl2−/− placentas display phenotypic hallmarks of Spint1 deficiency**

Spint1 encodes a cell membrane-associated Kunitz type 1 serine protease inhibitor (Shimomura et al., 1997) that is expressed in several epithelial cells and tissues, including the placenta, kidney and intestine (Kataoka et al., 2000; Yamauchi et al., 2004; Oberst et al., 2005; Szabo et al., 2007; Kawaguchi et al., 2011). Similar to Grhl2−/− placentas, choricorial trophoblasts of Spint1−/− placentas fail to establish a labyrinth, fetal vascularization is absent, and the trophoblodt layer appears to be compact (Tanaka et al., 2005; Fan et al., 2007; Szabo et al., 2007). Moreover, SPINT1 is expressed at high levels in BCT cells in E9.5 placentas (Tanaka et al., 2005; Fan et al., 2007; Szabo et al., 2007). Interestingly, the Spint1 gene featured a GRHL2 ChIP peak at the 3′ end in a region that contains the grainyhead consensus motif CAGCAAGTT (Fig. 5A) and that displayed histone H3 lysine 4 monomethylation (H3K4me1) and histone H3 lysine 27 acetylation (H3K27ac) in mouse placenta, two histone modifications characteristic of active enhancers (based on mouse placenta ENCODE data, not shown).

Together, these data suggest that GRHL2 might serve to ensure high Spint1 expression levels in BCT cells. The downregulation of Spint1 mRNA in total placenta extracts of Grhl2−/− deficient mice was relatively mild (Fig. 5C). However, this might reflect a dilution of the cell-specific effect in BCT cells, since Spint1 is expressed at high levels in BCT cells and at lower levels in surrounding trophoblasts. To assess the effect of Grhl2 deletion on Spint1 expression in BCT cells, we localized Spint1 mRNA and protein in the placenta. Spint1 mRNA expression, as detected by in situ hybridization, showed a high level of Spint1 mRNA in BCT cells of wild-type placentas. In Gr hl2-deleted placentas, Spint1 mRNA was specifically reduced in BCT cells compared with controls (Fig. 6A-D). Next, we carried out immunofluorescence staining using a SPINT1 antibody, confirming high cell membrane-associated SPINT1 levels in BCT cells of control placentas with markedly lower expression in other trophoblast layers (Fig. 6E; supplementary material Fig. S7A,B). By contrast, BCT cells of Grhl2−/− placentas showed strongly reduced SPINT1 protein expression, which was at levels indiscernible from that in other surrounding cell types (Fig. 6F; supplementary material Fig. S7C,D). We quantitatively estimated SPINT1 immunofluorescence intensity in Grhl2-deficient versus control BCT cells and overlying choricorial trophoblast cells using software-assisted (ImageJ) image analysis. This revealed a specific and significant (P<0.001) reduction of SPINT1 immunofluorescence intensity in the BCT cells of Grhl2−/− placentas, which resembled the weaker staining intensity of surrounding trophoblasts (Fig. 6G). These results supported the hypothesis that GRHL2 functions to enhance Spint1 expression levels in BCT cells.

**Spint1−/− mice have been shown to exhibit reduced Cdhl expression in BCT cells as well as basement membrane defects, including fragmented laminin deposition and disrupted basement membranes at the chorioallantoic interface adjacent to the BCT layer (Fan et al., 2007; Szabo et al., 2007). We analyzed E9.5 Grhl2−/− placentas for these phenotypic hallmarks of Spint1 deficiency. In wild-type placentas the BCT cells formed a single compact epithelial-like cell layer and expressed high levels of CDH1, which lined cell junctions. By contrast, in Grhl2−/− deleted BCT cells CDH1 expression was strongly reduced (Fig. 6H). Immunofluorescence staining for the basement membrane markers laminin and collagen IV revealed continuous basement membranes at the chorioallantoic interface lining the basal surface of BCT cells in controls (Fig. 6I,J), whereas in Grhl2 mutants the basement membranes were fragmented and appeared at atypical positions relative to the BCT cell layer (Fig. 6K,M). Transmission electron microscopy confirmed the fragmentation and thinning of basement membranes in Grhl2−/− placentas (Fig. 6N-Q).

Each of these phenotypes was strongly reminiscent of that reported in Spint1−/− placentas, consistent with the hypothesis that they are caused by the reduction of SPINT1 expression in Grhl2−/− placentas. Importantly, immunostaining showed that SPINT1 (supplementary material Fig. S8A,B), CDH1 (supplementary material Fig. S8C,D) and laminin and collagen IV (supplementary material Fig. S8E-H) expression levels and distribution patterns were normal in BCT cells of Sost2Cre;Grhl2−/− placentas, excluding an effect of extraplacental GRHL2 in the above phenotypes.

**Evidence for conserved GRHL2 expression and activity**

To determine the potential relevance of GRHL2 and its target gene set in human placentation, we examined GRHL2 expression in human placentas by immunohistochemistry (Fig. 7A-E). GRHL2 was predominantly expressed in human villous cytrophoblasts, but also in some STB cells of primary stem villi adjacent to the choricorial plate and in distal villi (Fig. 7A). GRHL2 expression in human villous cytrophoblasts was reminiscent of the pattern in mouse BCT cells. Moreover, GRHL2 was present in invasive extravillous trophoblasts and in cytrophoblast cell columns (Fig. 7B,C). During later pregnancy, GRHL2 was present at high levels also in most STB cells. No GRHL2 staining was observed in the mesenchymal cells of the villous stroma or in vascular cells (Fig. 7D,E).
We next analyzed the placental expression domain of human homologs of the Grhl2 target gene set using publicly available high-quality immunohistochemistry data deposited in the Human Protein Atlas Project (Uhlen et al., 2010). To avoid the detection of non-specific staining, we limited the analysis to antibodies with supportive western blot data and medium or high staining in placentas based on Protein Atlas scoring, which were available for KCNK1, RAB15, LAD1 and TFAP2A. Remarkably, each of these proteins was co-expressed with GRHL2 in villous cytotrophoblasts and STB cells (supplementary material Fig. S9).

Additionally, based on publically deposited microarray data from 34 human placenta samples (Huuskonen et al., 2008; Mikheev et al., 2008; Founds et al., 2009), human orthologs of GRHL2 and its targets showed a statistically significant co-regulation (P<0.01; Fig. 7F). Within the target gene set, SPINT1 expression displayed one of the highest correlations with GRHL2 (r=0.94). To further substantiate their co-regulation, we analyzed GRHL2 and SPINT1 mRNA expression in 84 human villous placenta samples by quantitative PCR and found a high level of correlation across all samples (r=0.77, P<10^-6; Fig. 7G). Immunohistochemistry confirmed previously reported data that SPINT1 is highly expressed in villous cytotrophoblasts of primary stem villi and more distal villi (Fig. 7H,I) (Hallikas et al., 2006). Collectively, these data suggest that the Grhl2-dependent target gene set that we identified in mouse is conserved in human placentation. The findings also indicate that GRHL2 targets a villous cytotrophoblast signature and are consistent with the notion that human villous cytotrophoblasts share functional characteristics with BCT cells in mice.

DISCUSSION

Our findings indicate that Grhl2 is essential for normal placental development as well as for appropriate gene regulation and differentiation of BCT cells of the placental labyrinth. GRHL2 transcriptionally activates regulators of placental development and epithelial morphogenesis, including Spint1, which encodes the serine protease inhibitor Kunitz type 1 that regulates BCT cell integrity and labyrinth formation. Spint1 might mediate some of the effects of GRHL2, since Grhl2-deficient placentas displayed characteristic hallmarks of Spint1 deficiency. In human placentas, orthologs of GRHL2 and its targets displayed co-regulation, suggesting conservation of the Grhl2-coordinated gene network and that these genes might participate in syndromes of placental dysfunction.

This is the first report to identify a role of Grhl2 in placenta development and in branching morphogenesis in general. In
addition, we identify genome-wide direct and indirect targets of *Grhl2*, which are enriched in genes encoding regulators of epithelial cell morphogenesis and placenta development. Our data support the hypotheses that GRHL2 acts to transactivate, rather than to transrepress, these target genes and functions to enhance SPINT1 levels in BCT cells, thereby ensuring BCT layer integrity. Moreover, our results indicate that human villous cytotrophoblasts express human orthologs of GRHL2 and several of its targets, lending further support to the concept that these cells exhibit cellular and molecular similarities with mouse BCT cells.

*Grhl2* is expressed in several trophoblast cell types, including BCT cells, spongiotrophoblasts and TGCs. In *Grhl2*−/− placentas, each of these cell types lacks *Grhl2* expression, complicating the interpretation of their relative contribution to the phenotype. Several pieces of evidence suggest that *Grhl2* deletion in BCT cells is primarily responsible for the branching phenotype observed. First, BCT cells are known to be central initiators of labyrinth branching morphogenesis, and these cells exhibit defects in cell polarity, basement membrane deposition, and *Spint1* and *Cdh1* expression following *Grhl2* deletion. It is known that the interaction of the basement membrane component laminin with cell surface integrin receptors of the adjacent epithelium is crucial in the regulation of three-dimensional epithelial morphogenesis (Klein et al., 1988; Sorokin et al., 1990; Durbeej and Ekblom, 1997). Importantly, previous studies have revealed that structural defects of basement membranes at the chorioallantoic interface of collagen IV (*Col4a1/Col4a2*)-mutant mice resulted in impaired placental labyrinth formation (Pöschl et al., 2004). Moreover, disruption of *Cdh1* expression is associated with defective labyrinth development (Stemmler and Bedzhov, 2010). Thus, the defects of BCT cells in *Grhl2* mutants reasonably explain the observed failure of labyrinth formation. By contrast, *Grhl2* mutants initially displayed normal spongiotrophoblast and TGC formation based on marker gene expression analysis. Furthermore, *Spint1* displays high expression levels only in BCT cells, but not in other trophoblast cell types, and its expression is strongly reduced only in BCT cells of *Grhl2* mutants. Together, these results suggest that *Grhl2* activity in BCT cells is crucial for labyrinth morphogenesis. However, we cannot exclude additional defects in other trophoblast cell types that might in part be obscured by the early and severe placenta phenotype of mutants. *Grhl2* deletion in specific trophoblast cell types would be required to clarify the cell type-specific effects of *Grhl2* in more detail.

As outlined above, several pieces of evidence support the notion that *Spint1* deficiency contributes to the placental phenotype of *Grhl2*−/− mice. However, ultimate proof of a crucial role of *Spint1* downstream of *Grhl2* is lacking, as it would require a rescue of *Spint1* expression or genetic interference with the *Spint1/prostasin/matriptase* cascade in a *Grhl2*−/− setting, for example by additionally deleting *Pross8* or *St14* (Lin et al., 1999; Szabo et al., 2007, 2012). Both, *Grhl2* and *SPINT1* are expressed in human villous cytotrophoblasts (Shimomura et al., 1997; Kataoka et al., 2000; Wilanowski et al., 2002; Hallikas et al., 2006; Szabo et al., 2007). Similar to BCT cells, the villous cytotrophoblasts are in direct contact with the basement membrane that separates them from mesodermal fetal stroma and blood vessels. Defects of trophoblast differentiation and morphogenesis are linked with pregnancy-associated diseases. Together with our finding that the expression of *GRHL2* and that of its target genes (including *SPINT1*) are closely correlated in human placenta specimens, this raises the possibility of an involvement of *GRHL2* and its target gene set in human placenta, a hypothesis that merits future detailed analyses.

A comparison of our GRHL2 ChIP-seq data with GRHL2 ChIP-seq data recently generated using primary human airway lung epithelial cells in culture (Gao et al., 2013) and kidney epithelial cells (mIMCD-3; our unpublished data) showed a peak overlap of 17.2% and 41%, respectively. Our findings suggest that GRHL2 controls a set of genes that are generally important for epithelial cell development and thus are common for different cell types or tissues.
but at the same time identify placenta-specific GRHL2 targets in mice. Consistently, ontology analysis of genes with GRHL2 binding peaks revealed a substantial overrepresentation of genes involved in epithelial cell development and morphogenesis and placenta development. Our finding that GRHL2 preferentially associates with genes that are downregulated in Grhl2−/− placenta, but not with those that are upregulated, provides in vivo evidence that GRHL2 might preferentially activate rather than repress the transcription of its direct target genes.

We have previously identified Cdh1 as an embryonic GRHL2 target and provided evidence for a direct mechanism of regulation through association of GRHL2 with intron 2 of Cdh1 (Werth et al., 2010). Although GRHL2 binding to Cdh1 intron 2 has been confirmed in several studies using ChIP on epiblast-derived cell types (Werth et al., 2010; Varma et al., 2012; Gao et al., 2013), we failed to detect GRHL2 binding at the Cdh1 locus in placenta tissues. Furthermore, a previous study has shown that Cdh1 intron 2 sequences are dispensable for Cdh1-specific reporter gene expression in extraembryonic, but not intraembryonic, tissues (Stemmler et al., 2005). Together, these findings suggest that Cdh1 expression in extraembryonic trophoblast cells occurs independently of GRHL2 association with intron 2 and instead might involve GRHL2-dependent regulation of Spint1. These ideas require confirmation in future studies.

The expression of Cldn4, as another previously identified embryonic GRHL2 target (Werth et al., 2010), was analyzed in whole placental extracts and in BCT cells. Cldn4 was robustly expressed in BCT cells, as detected by in situ hybridization and immunofluorescence staining, but we found no profound effect of Grhl2 deficiency on placental Cldn4 expression. Consistently, Cldn4 was not represented within the Grhl2 core target set that we identified by integrating comparative microarray analysis of control versus Grhl2 mutant placentas and ChIP-seq. This points to alternative pathways maintaining Cldn4 expression in BCT cells.

Thus, neither Cldn4 nor Cdh1 seems to be a direct Grhl2 target gene in BCT cells of the mouse placenta. This argues for a marked tissue specificity of Grhl2 functions and suggests that the target gene set of Grhl2 in the placenta differs from that in other tissues.

Placentas from globally Grhl2-deficient mice displayed a specific defect of labyrinth formation that was not seen in placentas from mice with an epiblast-specific deletion of Grhl2. However, epiblast-specific Grhl2 deletion failed to rescue embryonic lethality, indicating that intraembryonic Grhl2 is essential for survival past E11.5. The cause of this lethality is currently unknown. Defects of neural tube closure are normally consistent with survival to later stages of embryonic development. Mice with the mutant allele Grhl2Δ−/− (Pyrgaki et al., 2011) have been reported to occasionally survive to E18.5 and then display heart defects, with a thinning of the ventricular walls and expansion of the ventricles. Hence, it appears possible that heart defects might have contributed to the embryonic lethality of Sox2Cre;Grhl2Δ−/− mice, but we have not analyzed this in detail. Defects in other vascular and hematopoietic lineages could also potentially explain the embryonic lethal phenotype.

The severe placental defects in Grhl2-deficient mice are likely to eventually compromise embryonic development; for example, knockout of Rbm15 or Socs3 manifests placental defects of somewhat comparable severity to those of Grhl2 mutants (Takahashi et al., 2003; Raffel et al., 2009). In these mice, the progressive placental defects are associated with embryonic death starting at E10.5. When placental defects are rescued, they survive to birth. Since epiblast-specific deletion of Grhl2 rescued placental development, but not embryonic survival, we cannot definitively prove the requirement for placental Grhl2 in maintaining embryonic survival. This would require placenta-specific deletion of Grhl2. We attempted to generate Grhl2-deficient tetraploid blastocysts in order to carry out tetraploid complementation with wild-type embryonic stem cells. However, we were unable to achieve this because tetraploid blastocysts generated from embryos originating from intercrosses of Grhl2−/− mice, regardless of genotype, failed to efficiently support embryonic development.

We believe that our findings may have translational implications, as we have pursued a clinical line of investigation including human samples, and will be of relevance to placental biologists and to researchers exploring the mechanisms and consequences of placental malfunction.

**MATERIALS AND METHODS**

**Animals**

Generation of the Grhl2-deficient alleles Grhl2Δ−/− and Grhl2Δ+/− has been described (Werth et al., 2010). Mice with conditional Grhl2 alleles were obtained from embryonic stem cells containing a Grhl2 gene with exon 4 flanked by two loxP sites (supplementary material Fig. S5A). Breeding of female Grhl2Δ+/Δ males carrying the Sox2Cre transgene (Sox2Cre;Grhl2Δ−/−) generated Sox2Cre;Grhl2Δ−/− mice (Hayashi et al., 2002). For further details, see supplementary material methods and Tables S4 and S5.

**Human tissue samples**

We included non-labored placenta samples from complicated and uncomplicated pregnancies delivered by elective Cesarean section at Oslo University Hospital, Oslo, Norway, to analyze the expression correlation of GRHL2 and SPINT1. Details are provided in the supplementary material methods.

**Electron microscopy**

The fixation and imaging of E9.5 placentas are described in the supplementary material methods.

**Immunofluorescence staining**

Tissues were fixed in PBS containing 4% paraformaldehyde for 1 h at 4°C and embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek) for cryosectioning. Primary antibodies were incubated overnight at 4°C. For further details of immunofluorescence staining, including antibodies, confocal microscopy and analysis of fluorescence intensity, see the supplementary material methods.

**GRHL2 immunohistochemistry**

Immunohistochemistry was performed using the EnVision System-HRP (AEC) Kit for rabbit primary antibodies (DakoCytomation). For details, see the supplementary material methods.

In situ hybridization

In situ hybridization on placenta tissue sections was carried out as described in the supplementary material methods and Table S6.

**RNA extraction, cDNA synthesis and real-time PCR**

Total RNA was isolated using the RNeasy Mini Kit (Qiagen) and cDNA synthesis was performed with the RevertAid First-Strand cDNA Synthesis Kit (Fermentas). Real-time PCR was performed using MESA GREEN qPCR MasterMix Plus for SYBR Assay Rox (Eurogentec) and primers at 200 nM final concentration each. For primer sequences and further details, see the supplementary material methods and Tables S7 and S8.

**Microarray analysis**

Differential gene expression was examined by Illumina Mouse WG-6 v2.0 Expression Bead Chip microarray analysis. The extraembryonic trophoderm-derived tissues (chorion, ectoplacental cone and giant cell layer) at E7.5 and the visually identified placentas at E9.5 were separated from embryonic tissue and maternal uterus.
The publicly available human placenta data were downloaded from GEO under accession numbers GSE9984, GSE12767 and GSE7434 (Huuskonen et al., 2008; Mikheev et al., 2008; Founds et al., 2009). For details of the microarray and data analysis, see the supplementary material methods.

ChiP

Twenty-five C57BL/6J E17.5 mouse placentas were harvested, maternal uterine tissue was removed and placentas were pooled. Cross-linking was performed with 1% formaldehyde in PBS for 15 min at room temperature. Chromatin was fragmented to an average size of 300-500 bp by sonication. 100 µg chromatin and 5 µg GRHL2 antibody or isotype control antibody (rabbit IgG) as background control were used per assay using a Chromatin Immunoprecipitation Assay Kit (Upstate, Millipore) according to the manufacturer’s protocol. For ChIP-PCR, nine E9.5 placentas were harvested, pooled and cross-linked with 1% formaldehyde for 20 min at room temperature. Sonication of chromatin produced DNA fragments of average size 200-300 bp. 25 µg chromatin and 6 µg GRHL2 antibody or rabbit IgG as background control were used per assay according to established protocols (Li et al., 2003; Schmidt et al., 2009).

ChiP-seq

Complex-associated DNA fragments were de-cross-linked and subjected to a standardized ChiP-sequencing pipeline (Solexa). Libraries of 250-350 bp were prepared according to standard Illumina protocols. For details of the ChiP-seq data analysis, see the supplementary material methods. Microarray and ChiPseq data are available at GEO under the accession numbers GSE65963 (reference series), GSE65960 (microarray data) and GSE65962 (ChiPseq data).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

K.M.S.-O. and K.W. designed the project. K.W. performed most of the experiments, with the support of E.Po., M.W., A.A., M.B. and E.P. C.H. and C.D. performed integrated bioinformatic analyses. K.M.S.-O. and K.W. helped with the generation of the conditional Grhl2 allele. S.V., R.M. and D.W. helped with ChIP-seq data analysis and data interpretation.

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