The Hippo pathway component Wwc2 is a key regulator of embryonic development and angiogenesis in mice

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Abstract
The WW-and-C2-domain-containing (WWC) protein family is involved in the regulation of cell differentiation, cell proliferation, and organ growth control. As upstream components of the Hippo signaling pathway, WWC proteins activate the Large tumor suppressor (LATS) kinase that in turn phosphorylates Yes-associated protein (YAP) and its paralog Transcriptional coactivator-with-PDZ-binding motif (TAZ) preventing their nuclear import and transcriptional activity. Inhibition of WWC expression leads to downregulation of the Hippo pathway, increased expression of YAP/TAZ target genes and enhanced organ growth. In mice, a ubiquitous Wwc1 knockout (KO) induces a mild neurological phenotype with no impact on embryogenesis or organ growth. In contrast, we could show here that ubiquitous deletion of Wwc2 in mice leads to early embryonic lethality. Wwc2 KO embryos display growth retardation, impaired vascularization, and finally embryonic death. A whole-transcriptome analysis of embryos lacking Wwc2 revealed a massive deregulation of gene expression with impact on cell fate determination, cell metabolism, and angiogenesis. Consequently, a perinatal, endothelial-specific Wwc2 KO in mice led to disturbed vessel formation and vascular hypersprouting in the retina. In summary, our data elucidate a novel role for Wwc2 as a key regulator in early embryonic development and sprouting angiogenesis in mice.

Introduction
Mammalian embryogenesis is a highly regulated process that includes specification of cell fate, targeted cell migration, and controlled cell proliferation. Altered expression or mutations of crucial genes can lead to a disturbed cell differentiation, deregulated organ development, impaired angiogenesis, and finally embryonic lethality.

Determination of cell fate starts very early in embryonic development. Mouse embryos at the blastocyst stage consist of two different cell types, (i) the inner cell mass (ICM) that later forms the embryo as well as extra-embryonic structures (e. g. the yolk sac) and (ii) an surrounding layer of trophodermal cells (TE) that will give rise to the embryonic portion of the placenta. The Hippo signaling pathway and its key components, the co-transcription factor Yes-associated protein (YAP) and its paralog Transcriptional coactivator-with-PDZ-binding-motif (TAZ), are crucial for early embryogenesis as they determine cell-type-specific gene expression in the TE and the ICM. In the TE cells, the Hippo pathway is silent which results in an enhanced activity of nuclear YAP/TAZ. In contrast, in cells of the ICM, Hippo pathway activity is high preventing YAP/TAZ-mediated gene transcription.
Besides its role in cell fate determination during embryogenesis, the highly conserved Hippo signaling pathway is known to regulate postnatal organ growth in all organisms from fly to man\textsuperscript{8–10}. In mammals, impaired Hippo signaling results in nuclear import of YAP/TAZ, inhibition of apoptosis, and enhanced cell proliferation. Nuclear YAP activity depends on its phosphorylation status, which prevents transport of YAP/TAZ into the nucleus and marks these proteins for proteasomal degradation\textsuperscript{8,10}. These events are controlled by a growing number of upstream effectors and a cytosolic core kinase cascade culminating in YAP/TAZ phosphorylation by the Large tumor suppressor (LATS) kinases 1 and 2\textsuperscript{11,12}. The family of WWC proteins, composed of WWC1 (also called kidney-and-brain (KIBRA) protein), WWC2 and WWC3, have a crucial impact on Hippo signaling because they enhance LATS1/2 kinase activity and subsequently YAP/TAZ phosphorylation\textsuperscript{13,14}. The structural similarity and a common subset of interacting partners indicate a redundant function of the WWC proteins\textsuperscript{14,15}. Consequently, a ubiquitous knockout (KO) of \textit{Wwce}1 expression in mice that have lost the WWC3 gene during evolution leads to an only mild neurological phenotype but has no crucial impact on embryogenesis or organ development likely due to Wwc2-mediated compensation\textsuperscript{16,17}. In contrast, a hepatocyte-specific double KO of both \textit{Wwce}1 and \textit{Wwce}2 in mice results in Hippo pathway-dependent organ overgrowth and subsequent liver carcinoma\textsuperscript{14}.

Recent data have shown that murine \textit{Wwce}2 is already expressed in the blastocyst, whereas \textit{Wwce}1 expression is almost undetectable at this stage\textsuperscript{18}. To investigate a putative \textit{Wwce}2-specific function in early embryogenesis, we generated a ubiquitous deletion of \textit{Wwce}2 in mice and observed embryonic lethality around day E11.5. Analysis of \textit{Wwce}2 KO embryos revealed growth retardation effects, an impaired embryonic vessel formation and a disturbed vascularization within the placenta. A whole-transcriptome analysis of \textit{Wwce}2 KO embryos at E11.5 demonstrated a strong deregulation of gene expression with an impact on Hippo signaling, cell metabolism, and vascular development. Furthermore, an inducible and endothelial-specific \textit{Wwce}2 KO in perinatal mice led to impaired vessel formation and vascular hypersprouting in the retina indicating a crucial role for \textit{Wwce}2 in angiogenesis.

**Results**

**Embryonic lethality in \textit{Wwce}2 KO mouse embryos**

To analyze \textit{Wwce}2 function during embryonal development, a transgenic mouse line with a ubiquitous knockout of \textit{Wwce}2 was generated. For this, animals from a floxed \textit{Wwce}2 (\textit{Wwce}2\textsuperscript{fl/fl}) line\textsuperscript{14} were crossed with mice from a phosphoglycerate kinase (Pgk) 1 driven \textit{Cre} recombinase deleter line (Pgk-Cre), in which target genes can be ubiquitously inactivated from early embryonic stages on\textsuperscript{19}. The \textit{Wwce}2 genotype of the individual offspring (4 weeks old) was verified via PCR analysis (Supplemental Fig. S1A). From a total number of 83 analyzed mice, no animal with a homozygous \textit{Wwce}2 KO (\textit{Pgk-Cre-Wwce}2\textsuperscript{KO/KO}) could be found, but 28 wild-type (\textit{Wwce}2\textsuperscript{+/+}) and 55 heterozygous animals (\textit{Pgk-Cre-Wwce}2\textsuperscript{KO/+}). The last-named were vital and phenotypically normal just as \textit{Pgk-Cre}-negative \textit{Wwce}2\textsuperscript{fl/fl} mice. These findings pointed to intrauterine death of embryos lacking \textit{Wwce}2 expression. To determine the onset of embryonic lethality in more detail, we next isolated embryos at different developmental stages from embryonic day (E) 6.5 to E14.5 and determined their genotype. For this, \textit{Wwce}2\textsuperscript{fl/fl} animals were crossed with mice from a zona pellucida glycoprotein 3 (\textit{Zp}3) Cre recombinase deleter line (\textit{Zp}3-Cre), in which target genes can be inactivated from growing oocytes in a \textit{Zp}3 promoter-dependent manner\textsuperscript{20}. Normal Mendelian frequencies of wild-type (\textit{Wwce}2\textsuperscript{+/+}), heterozygous (\textit{Zp}3-Cre-\textit{Wwce}2\textsuperscript{KO/+}) and homozygous (\textit{Zp}3-Cre-\textit{Wwce}2\textsuperscript{KO/KO}) embryos were observed until E11.5 (Supplemental Tab. 3). Whereas \textit{Wwce}2\textsuperscript{+/+} and heterozygous \textit{Zp}3-Cre-\textit{Wwce}2\textsuperscript{KO/+} embryos showed normal development after E11.5, homozygous \textit{Zp}3-Cre-\textit{Wwce}2\textsuperscript{KO/KO} embryos (called \textit{Wwce}2 KO later on) were degenerated or/ and resorbed (Supplementary Fig. S1B). A Western blot analysis confirmed the lack of WWC2 protein in the E11.5 \textit{Wwce}2 KO embryos (Supplementary Fig. S1C). To gain more insight into the putative function of \textit{Wwce}2 in embryogenesis, we investigated the spatial and temporal expression of \textit{Wwce}2 during normal embryonic development. As immunohistochemical studies were not possible for WWC2 due to the lack of suitable antibodies, we used whole-mount in situ hybridization (WISH) to detect \textit{Wwce}2 mRNA in wild-type mouse embryos between E7.5 and E11.5. \textit{Wwce}2 transcripts were ubiquitously detectable at E7.5, in both embryonic and extra- embryonic tissues (Fig. 1A). At E8.5, \textit{Wwce}2 expression was mainly visible in mesenchymal tissues (Fig. 1B). In E10.5 and E11.5 embryos, \textit{Wwce}2 mRNA was detectable in the surface ectoderm, with domains of enrichment in the branchial arches and the posterior-distal region of the limb anlagen (Fig. 1C, D). In addition to superficial ectodermal expression, \textit{Wwce}2 expression was found in the roof plate of the neural tube and the dorsal root ganglia (Fig. 1C, D). To get a more quantitative analysis of \textit{Wwce}2 mRNA expression during embryogenesis, we isolated whole mRNA from embryos at different stages and performed quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). This approach revealed that \textit{Wwce}2 expression is already present at E8.5 and that the expression level remains stable until E13.5 with only minor changes (Supplementary Fig. S2).
Placenta defects in Wwc2 KO embryos

The WISH results provide novel insight into the developmental regulation of Wwc2 expression. However, the data did not explain the reason(s) for the embryonic lethality observed in Wwc2 KO mice. As embryonic death around E11.5 in mice is typically linked to placental defects and a disturbed nutrition of the embryo, we next analyzed the structure of the placenta from control (Wwc2+/− or Zp3-Cre-Wwc2+/−) and Wwc2 KO embryos, respectively. Murine placenta is composed of the maternal decidua (MD), a single layer of trophoblast giant cells (TGC), a layer of embryonic spongiotrophoblasts (STL) and the labyrinth system (LA) that ensures the exchange of nutrients and oxygen between maternal and fetal vessels. PAS staining of placenta sections from embryos at E11.5 revealed an unorganized structure of the anatomical layers in Wwc2 KO embryos. The TG layer, normally arranged in a chain-like structure separating the MD and the STL, was dispersed and hardly visible (Fig. 2A). In addition, the STL was thickened in the Wwc2 KO placenta compared to the control, whereas the LA appeared more compact (Fig. 2A).

Vascularization within the labyrinth system is a highly regulated process ensuring the metabolic requirements of the embryo with a supply of essential components from maternal blood. Maternal and fetal blood vessels in the labyrinth system are distinguishable by the presence of different types of erythrocytes. Maternal erythrocytes (ME) appear as small, non-nucleated cells, whereas fetal erythrocytes (FE) are bigger and nucleated. Hematoxylin and eosin (H&E) staining of placenta sections from E11.5 Wwc2 KO embryos revealed a markedly reduced number of fetal blood vessels and FE in the LA layer (Fig. 2B, C). Furthermore, an immunofluorescence staining of the placental vasculature using antibodies directed against the endothelial marker Platelet/endothelial cell adhesion molecule-1 (PECAM1) and the trophoblast cell marker cytokeratin (CK) indicated a failure of embryonic vessels to permeate into the STL of the Wwc2 KO placenta (Fig. 2D).

Wwc2 deficiency leads to an impaired angiogenesis in the yolk sac and embryo proper

The observed defects in the placental structure of Wwc2 KO embryos are likely to cause an insufficient supply of the embryo with nutrients and oxygen that finally leads to embryonic death around E11.5. To test whether Wwc2 ablation leads to additional defects in the embryo itself, a tetraploid complementation assay was performed. In this assay, extraembryonic tissue is formed by tetraploid cells, while the embryo is exclusively formed by diploid embryonic stem (ES) cells. Using wild-type tetraploid cells to ensure normal formation of extraembryonic tissue and ES cells from three different lines were used (Supplementary Table 4). These data indicated extra-placental, embryo-specific defects in development that are linked to a Wwc2 ablation.

Next, we used Wwc2-deficient embryos between E9.5 and E11.5 for histological examination. Compared to controls, Wwc2 KO embryos appeared growth retarded and less developed at E9.5, E10.5, and E11.5. Furthermore, in the absence of Wwc2, an almost complete loss of blood-filled vessels was observed in the embryos and the yolk sac at E11.5 (Fig. 3).

The vascular structure of Wwc2 KO embryos at E11.5 was further analyzed using whole mount immunostaining for PECAM1-positive, endothelial cells. This approach revealed that the vascular network was present but less organized in the absence of Wwc2 (Fig. 4A). Cranial vessels in the Wwc2 KO embryo showed an almost uniform diameter and resembled a honeycomb-like structure with no large vessels, which were present in the control embryos at this time point (Fig. 4B). A co-immunostaining of endothelial cells (PECAM1-positive) and pericytes, multifunctional mural cells wrapping around E11.5 in mice is typically linked to placental defects and a disturbed nutrition of the embryo, we next analyzed the structure of the placenta from control embryos at E11.5 revealed an unorganized structure of the anatomical layers in Wwc2 KO embryos. The TG layer, normally arranged in a chain-like structure separating the MD and the STL, was dispersed and hardly visible (Fig. 2A). In addition, the STL was thickened in the Wwc2 KO placenta compared to the control, whereas the LA appeared more compact (Fig. 2A).

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around blood capillaries (positive for the marker protein smooth muscle actin (SMA)), revealed an irregular patterning of these cell types in the vascular system of \textit{Wwc2} KO embryos (Fig. 4C). Furthermore, the architecture of the vascular system surrounding the neural tube (NT) was disturbed and the lateral branches between the intersomitic vessels were almost completely absent in the \textit{Wwc2} KO embryo (Fig. 4C). Similar to the embryo trunk, the yolk sac of the \textit{Wwc2} KO embryos lacked the typical hierarchical tree-like structure of the vascular network, instead vessels were organized in a more primitive honeycomb-like network (Fig. 4D).

**Whole-transcriptome analysis of \textit{Wwc2} KO embryos**

To elucidate the function of \textit{Wwc2} in embryonic development at the molecular level, a whole-transcriptome analysis by next generation sequencing was performed. In order to identify genes that are differentially expressed, whole RNA was isolated at E11.5 from wildtype (\textit{Wwc2}^{+/+}; \(n=3\)), heterozygous (\textit{Zp3-Cre-Wwc2^{KO/+}}; \(n=3\)), and homozygous (\textit{Zp3-Cre-Wwc2^{KO/KO}}; \(n=4\)) embryos and the mRNA levels were determined. The FPKM (fragments per kilobase of transcript per million fragments mapped) value was used to normalize the number of reads to the mRNA length and the total read number in the measurement. With this normalization, mRNA levels can be compared in different samples taking both molar concentration and transcript length into account. The reads in the measurement allowed the identification of a total number of 20,653 genes. No significant differences within the transcriptome between wild type and heterozygous \textit{Wwc2} KO embryos were detected (data not shown). In contrast, the transcriptomes obtained from the homozygous \textit{Wwc2} KO embryos displayed remarkable changes when compared to the wildtype and heterozygous embryos (Fig. 5A).
expected, the group of differentially regulated genes (DEG) included a numerous known YAP/TAZ target genes that were upregulated in homozygous Wwc2 KO embryos (Fig. 5A). For an in-depth analysis, a threshold was set in which only the differentially expressed genes could be identified including 1362 upregulated and 622 downregulated genes (Fig. 5B). Scale bars represent 500 µm.

Fig. 3 Growth retardation and disturbed blood flow in Wwc2 KO embryos and yolk sacs. Wwc2 KO embryos at E9.5 or E10.5 appeared growth retarded and less developed when compared to control embryo. Furthermore, no blood-filled vessels are observed in Wwc2 KO embryos (E10.5) and yolk sacs (E11.5). Scale bars represent 500 µm.

involved in DNA repair, protein degradation, and tissue remodeling. Among the 30 most downregulated genes, a remarkable number of genes have functions in the nervous system (Supplementary Tab. 6).

For further interpretation of the results, the annotation tool software from the Database for Annotation, Visualization, and Integrated Discovery (DAVID) was used to perform gene set enrichment analysis on Gene Ontology (GO) terms from the Uniprot “Tissue” or the “Biological Processes” data set to identify significantly enriched gene sets. The significantly upregulated genes could be significantly enriched in the three terms “Trophoblast stem cells”, “placenta” and “kidney” (Supplementary Fig. 3A). The significantly downregulated genes could be assigned to terms related to different brain regions (Supplementary Fig. 3A).

The gene set enrichment analysis for the ontology category “Biological Process” revealed that the upregulated genes in Wwc2 KO embryos were enriched in the terms “angiogenesis”, “transcription and translation”, “metabolism”, “placenta development”, and “cell adhesion” (Supplementary Fig. 4A). The downregulated genes in Wwc2 KO embryos were enriched in terms related to processes within the nervous system, e.g. “neuronal action potential” (Supplementary Fig. 4B).

Gene set enrichment analysis (GSEA), an alternative software tool for the analysis on differentially expressed genes, gave similar results when compared to the performed DAVID analysis (Supplemental Tables 7 and 8).

To confirm the transcriptome data indicating an effect of the Wwc2 KO on genes involved in Hippo signaling and angiogenesis, we used qRT-PCR analysis with RNA isolated from E11.5 control and homozygous Wwc2 KO embryos. These experiments revealed an enhanced expression of the YAP target genes Krt19, Cyr61, and Ctgf in Wwc2 KO embryos that likely resulted from a decreased Hippo signaling (Fig. 5C). Furthermore, a significantly upregulation of the angiogenesis-related genes Pecam1, Vegfa, and Junb in the Wwc2 KO embryos was detected probably causing an imbalance in angiogenesis (Fig. 5C).

Postnatal angiogenesis in the retina of mice with an endothelial-specific Wwc2 KO

The data from the phenotypic analyses clearly revealed an impact of the ubiquitous Wwc2 KO on angiogenesis inducing an early embryonic lethality. To gain more insight into the endothelial-specific function of Wwc2 in postnatal angiogenesis, we next examined the growth of retinal vessels in the absence of Wwc2 expression. For this approach, we crossed Wwc2fl/fl mice with mice from a Cadherin 5 (Cdh5)-CreERT2 line in which endothelial-specific activity of Cre recombinase is induced by Tamoxifen treatment35. To exclude a putative
Fig. 4 (See legend on next page.)
compensatory effect of Wwc1 in the absence of Wwc2 in endothelial cells, we included mice from a Wwc1fl/fl -Wwc2fl/fl line\(^{14}\) in these experiments.

Postnatally, Cre-mediated gene deletion was achieved through a triple intragastric Tamoxifen injection at P1–P3. Cre-negative animals were used as controls. At P6, mice were euthanized, and retinas were dissected for further analysis (Fig. 6A). Staining of retina flat mounts with Isolectin B4 (IB4) revealed a highly differentiated vessel network in control and Wwc2 KO retina. However, retinas from Wwc2 KO mice displayed an impaired vessel growth with hypsersprouting and increased vessel density at the vascular front (Fig. 6B).

Postnatal sprouting of vessels is controlled by a highly complex interplay between guiding tip cells and stalk cells\(^{36,37}\). Pathologic hypsersprouting is frequently connected to an increased number of tip cells\(^{38-40}\). Indeed, staining of retina with IB4 and antibodies against the tip cell marker Endothelial cell-specific molecule (Esm1)\(^{41}\) demonstrated an increased number of tip cells in the vascular front region in Wwc2 KO mice (Fig. 6C). A quantitative analysis of the observed retina phenotype revealed that the percentage of the vascular hyper-sprouting region in relation to the overall vascularized area was significantly higher in Wwc2 KO mice when compared to controls. Double KO (dKO) of both Wwc1 and Wwc2 did not further enhance this effect (Fig. 6D). Another evidence of vascular hypsersprouting in Wwc2 KO retinas was the significantly enlarged number of vessel endpoints in relation to the total vessel length in the analyzed images (Fig. 6E). Again, there was no difference between WWC2 KO and double KO animals.

**Discussion**

The Hippo signaling pathway is involved in the regulation of cell proliferation, apoptosis and organ growth. Besides the core kinase cascade, Hippo signaling is controlled through an increasing number of upstream regulators including the WWC proteins\(^{10-12}\). The WWC protein family was identified to induce LATS1/2 activity that promotes cytoplasmic retention of YAP in vitro\(^{13}\). Using KO mouse models, Wwc1 and Wwc2 were shown to have a Hippo pathway-dependent impact on postnatal organ growth and tumorigenesis. A hepatocyte-specific KO of Wwc1 and Wwc2, but not of a single WWC protein, leads to liver overgrowth and subsequent carcinoma formation in mice\(^{14}\). These findings confirm the important role of WWC proteins for Hippo signaling in vivo and furthermore suggest a redundant function of the different WWC family members.

Besides its role in control of postnatal cell proliferation, Hippo signaling is required for processes during embryogenesis. Consequently, gene KO in mice affecting the Hippo pathway frequently result in embryonic lethality and resorption that mainly occur between E8.5 and E13.5\(^{42-44}\). Mouse embryos harboring a deletion of Ww45 or Mst1/2 display vasculature defects and growth retardation as well as a disturbed placental development\(^{45,46}\).

Our report confirms the crucial role of the WWC gene family for Hippo signaling but further elucidate the specific role of Wwc2 in early embryogenesis.

A Wwc2 KO in mice leads embryonic lethality around day E11.5. Until this stage, embryonic morphogenesis, embryo segmentation, and organogenesis were present but in an impaired manner (Fig. 3). We speculate that Wwc2 deficiency is probably tolerated within very early steps of embryogenesis but become crucial between E8.5 and E11.5. During this embryonic period, the development of the murine placenta takes place that ensures the supply of the fetus with nutrients and oxygen from the mother\(^{26,28}\). Indeed, Wwc2 KO embryos showed a disturbed organization of placenta structures with impaired vessel formation and vascularization (Fig. 2). These placental defects seem to be an important, but not the exclusive reason for the observed embryonic lethality. Additionally, vascular development in the embryo proper and extraembryonic tissues was impaired. In control E11.5 embryos, cranial vasculature and the vessels in the yolk sac remodeled from a honeycomb-like structure to hierarchal tree-like structures with large and small blood vessels. However, vasculature in Wwc2 KO embryos was still honeycomb-like with all vessels of similar size indicating failure of remodeling. Since embryo and extraembryonic tissues are highly connected and inter-dependent, the impaired vascularization within the Wwc2 KO embryo might lead to disturbed blood circulation and (indirectly) to placental failure. Another scenario is based on defects in the placental vasculature that leads to malnutrition of the embryo, growth retardation and finally embryonic death. However, results of the tetraploid...
Fig. 5 (See legend on next page.)
complementation assay indicate that even in conditions, when placental development was normal, KO of Wwc2 in the embryo proper led to the death of the embryo. YAP was shown to be essential for the formation of the vascular system in mice. The vascular endothelial growth factor (VEGF) induces nuclear import and activation of YAP that results in enhanced transcription of the YAP target genes Ctgf and Cyr61 activating angiogenesis. Indeed, we demonstrated that deletion of Wwc2 leads to increased levels of Cyr61 and Ctgf, but also forces the expression of angiogenesis-related genes including Vegfa, Pecam1 and JunB (Fig. 5C).

Recent studies revealed that vessel remodeling and vascular sprouting are sensitive to a deregulation of the Hippo pathway and YAP-dependent gene expression. During the normal development of the murine vascular system, a honeycomb-like network of endothelial-lined, polygonal channels within the embryo and the yolk sac, and remodels to form a branched network of large and small vessels surrounded by smooth muscle cells. This process is described to be highly dependent on the expression of a variety of genes that are related to the Hippo pathway. In the Wwc2-deficient embryos and yolk sacs, vessels are arranged in the described honeycomb-like network without any hierarchical architecture indicating a developmental arrest and a failure of vessel remodeling. The altered distribution of vascular endothelial cells and pericytes surrounding the blood vessels strengthen these observations although Wwc2 appears to be dispensable for the differentiation of endothelial cells or pericytes from their precursor cells itself.

Unexpectedly, our transcriptome study revealed a strongly upregulated expression of placenta-specific genes in the Wwc2 KO embryo pointing to deregulation of cell fate determination at the preimplantation stage. In the blastocyst, proper differentiation of ICM and TE cell lineages depend on the expression of the transcriptional factor Cdx2 that in turn requires nuclear YAP activity. YAP is predominantly cytosolic in cells of the ICM, whereas in the TE cells, YAP displays a nuclear enrichment and induces the expression of genes associated with trophoblast specification including Cdx2. Consequently, activity of the core kinase cascade within the Hippo pathway is low in TE cells but high in the ICM preventing nuclear YAP accumulation. In Wwc2 KO embryos, we observed an upregulation of Cdx2 and Gata3 expression that points to a deregulation of the gene profile in ICM cells likely due to low LATS1/2 activity and accumulation of LATS1/2 activity venting its nuclear import and transcriptional activity. WWC2 was recently shown to prevent Angiomotins from pointing to a deregulation of the gene expression.

It is worth to note, that YAP activity at the blastocyst stage is also regulated by the Angiomotin (AMOT) family consisting of AMOT, Angiomotin-like protein 1 (AMOTL1) and Angiomotin-like protein 2 (AMOTL2). Angiomotins anchor YAP at the cytoskeleton preventing its nuclear import and transcriptional activity. WWC2 was recently shown to prevent Angiomotins from proteasomal degradation. Therefore, we speculate that loss of WWC2 expression does not only lead to decreased LATS1/2 activity but to lower Angiomotins levels both enhancing nuclear YAP activity in ICM cells. A ubiquitous Wwc2 KO led to various defects in development including a disturbed cell fate determination at the blastocyst state and deregulated vessel formation in the later steps of embryogenesis. In addition, our transcriptome approach and the gene set enrichment analysis of downregulated genes revealed an enrichment of GO terms related to the nervous system and brain development in Wwc2 KO embryos (Supplementary Figs. 3 and 4). Upregulated genes were enriched in GO terms related to glycolysis, hypoxia, regulation of gene expression, and protein translation in Wwc2 KO embryos (Supplementary Fig. 3). Most of these deregulated gene expression patterns might represent secondary effects caused by the disturbed angiogenesis and deficiencies in embryo nutrition. As a consequence, several survival mechanisms might be activated to compensate cell and tissue death and to prevent degeneration.

We propose that the impaired angiogenesis in Wwc2 KO embryos is likely caused by an inhibited Hippo pathway and an enhanced YAP/TAZ transcriptional activity in endothelial cells. This hypothesis is supported by our studies on postnatal vessel formation in the retina from mice with an endothelial-specific Wwc2 KO that
demonstrated an increased proliferation of tip cells and vascular hypersprouting (Fig. 6B, C). Tip cells are located at the ends of angiogenic sprouts and their filopodial protrusions directed vessel growth toward angiogenic attractants. Recent data showed that Cyr61, a known YAP/TAZ target gene, stimulates tip cell proliferation and activity. Therefore, loss of endothelial Wwc2 expression might activate YAP/TAZ-dependent Cyr61 expression that in turn leads to enhanced tip cell activity and disturbed vessel sprouting.

In summary, the results presented here elucidate the function of Wwc2 in embryogenesis and sprouting angiogenesis in mice and shed more light onto the role of the Hippo pathway on these processes. During the preparation of this manuscript, Virnicchi et al. presented data on the crucial role of Wwc2 on cell proliferation during pre-implantation of mouse embryos. Although their results partially differ from our data (likely due to the different experimental approach), this study further underlines the crucial role of Wwc2 in mouse embryogenesis.
Materials and methods

Mice

Animal experiments were performed in accordance with national guidelines and were approved by the local authorities of North Rhine-Westphalia, Germany.

Generation of mice with a floxed Wwc2 gene (Wwc2fl/fl) and mice harboring floxed Wwc2 and Wwc1 genes (Wwc1fl/fl-Wwc2fl/fl) were described before14. Wwc2fl/fl mice were crossed with Phosphoglycerate kinase (Pgk) promoter-driven Cre deleter mice to obtain ubiquitous Wwc2 deletion19. Crossing Wwc2fl/fl mice with mice from a Zona occludens protein 3 (Zp3) promoter-driven Cre deleter line was used to remove both maternal and zygotic Wwc2 expression20.

The Cadherin 5 (Cdhs5)-CreERT2 mouse line, enabling an inducible endothelial-specific activation of Cre expression, was described earlier35 and was a generous gift from R. Adams (Max-Planck-Institute for Molecular Biomedicine, Muenster, Germany). To generate a postnatal endothelial-specific knockout, mice were injected on postnatal day 1 (P1), P2, and P3 intragastric with 50 µl Tamoxifen (Sigma-Aldrich) at a concentration of 1 mg/mL in corn oil (Sigma-Aldrich).

Genotyping

Genotyping was performed as described earlier14. In brief, ear clips or embryonic tissues were lysed in DirectPCR-Tail Lysis buffer (Peqlab/VWR, Germany) containing Proteinase K (Qiagen) for 12 h at 56 °C. Proteinase K was heat inactivated for 5 min at 95 °C. Lysates were centrifuged at 14,000 × g for 10 min and the supernatants were used for genotyping by PCR analysis. The used primers are listed in Supplementary Tab. 1.

Generation of ES cell lines and tetraploid complementation assay

To establish embryonic stem (ES) cell lines, E3.5 blastocysts were flushed from uterus of Zp3-Cre-Wwc2fl/fl female mice mated with Wwc2fl/+ male mice and cultured on gamma-ray inactivated mouse embryonic fibroblasts with a standard procedure37 after removal of the zonae pellucidae by acidic Tyrode’s solution (Sigma-Aldrich). From 37 blastocysts collected, 20 ES lines were established. Seven of ES cell lines showed the homozygous Wwc2 ablation.

For the tetraploid complementation assay, two-cell embryos were flushed 42 h post-hCG from oviducts of mice with M2 medium and fused with a Cellfusion CF-150/B apparatus in a 250-µm gap electrode chamber (BLS Ltd., Budapest, Hungary) containing 0.3 M Mannitol with 0.1 mM MgSO4, 0.1 mM CaCl2, and 0.3% bovine serum albumin (Sigma-Aldrich). An initial electrical field of 2 V was applied to the embryos, followed by one peak pulse of 50 V for 35 µs. Embryos were washed and immediately transferred back into potassium simplex optimized medium (KSOM) medium62 in a 37 °C incubator. Fused tetraploid embryos were cultured overnight to four-cell stage and used for aggregation as reported with a slight modification63. Briefly, clumps of trypsin-treated ESCs (10–15 cells) were transferred into a dent in a drop of KSOM medium. Meanwhile, batches of 40–50 embryos were briefly incubated in acidified Tyrode’s solution until dissolution of their zonae pellucidae. Two embryos were placed in the dent next to cells to make one aggregate. All aggregates were assembled in this manner. After overnight culture at 37 °C in 5% CO2, blastocysts were transferred to each uterine horn of 2.5 days post coitum, pseudopregnant CD1 females that had been mated with vasectomized males. For cesarian derivation, recipient mothers were sacrificed at E19.5 and pups were quickly removed.

Retina isolation

Isolation of retinas from postnatal mice and preparation of flat mounts were performed according to a published protocol64.

Immunohistochemistry and immunofluorescence staining

Mouse embryos or placenta tissues were fixed in paraformaldehyde (PFA) for 4–24 h, washed with PBS, and embedded in Paraffin following standard procedures. Sections (4µm) were deparaffinized in Tissue-Clear (Sakura Finetek, USA) for 20 min, and rehydrated by passage through a decreasing alcohol gradient into distilled water. The sections were stained with hematoxylin and eosin (H&E) or periodic acid Schiff (PAS) stain following standard staining protocols. The primary antibodies used are listed in Supplementary Tab. 2. Antibodies were diluted in 1% bovine serum albumin (BSA) in PBS and incubated overnight. As secondary antibody the Vectashield ABC system (Vector Laboratories) was used with DAB (Peroxidase Substrate Kit, Vector Laboratories) as a substrate.

For immunofluorescence staining, embryonic tissues, or placenta were embedded in O.C.T. (Sakura Finetek) and immediately frozen in a bath of super-cooled 2-methylbutane. The sections (7 µm) were fixed with PFA, pre-treated with 0.2% Triton X-100 and blocked with 5% BSA in PBS for 1 h. Primary antibodies are listed in Table 2 of the supporting information. Antibodies were diluted in 1% BSA in PBS, applied to sections and incubated overnight at room temperature. To visualize antigens by immunofluorescence staining, the sections were washed with PBS and incubated with secondary antibodies coupled to fluorochromes (obtained from Invitrogen). Sections were analyzed using a fluorescence microscope (Zeiss, Observer Z1, HXP120, Axiocam MRm) and AxioVision 4.8 (Zeiss).
The whole mount immunostaining of PECAM1 in the embryos and yolk sac fixed in 4% PFA at 4°C overnight was carried out as described earlier. The vasculature in retinal flat mounts was visualized by IB4 staining. Tip cells were detected using antibodies against the ESM1 marker protein (Supplementary Table 2). For quantification of sprouting angiogenesis in the retina, digital images of the flat mounts were evaluated using Adobe Photoshop. Areas of normal vessel growth and hypersprouting were selected using the lasso and quick selection tools. Size of these areas measured in pixels was obtained from the histogram window. Quantitative analysis of the vascular network was performed in the selected areas using the free software AngioTool (National Institute of Health National Cancer Institute, Gaithersburg, MD).

Whole mount in situ hybridization

For WISH, embryos were processed according to the protocol provided in the MAMEP database (http://mamep.molgen.mpg.de). The probe for Wwc2 corresponds to nucleotides 2085–3230 of NM_133791.4. The probe template was produced by PCR with a reverse primer containing a T7 site for antisense transcription. A DIG-labeled probe was generated by in vitro transcription according to standard procedures, and staining was performed using BM Purple (Roche). Following the staining reaction, samples were postfixed and stored in 4% PFA/PBS. For imaging of WISH-stained embryos, a MZ16A dissection microscope (Leica) fitted with an AxioCam MRc5 (Carl Zeiss MicroImaging) was used in combination with the AxioVision Software (Carl Zeiss Micro-Imaging) for image processing.

RNA isolation and quantitative RT-PCR reaction

Total RNA was isolated from embryonic or placental tissue using the GenElute Mammalian total RNA Miniprep Kit according to the manufacturer’s instructions. cDNA was synthesized using the SuperScript III Reverse Transcriptase kit (Invitrogen). SYBR Green (Applied Biosystems) dye-based qRT-PCR was performed by the core facility genomics (University Hospital Münster, Germany) using CFX384 Touch (Biorad). The used primers are listed in Table 1 of the Supporting Information. Each cDNA sample was measured in triplicates and data are expressed relative to the housekeeping gene GAPDH. The samples from control and Wwc2 KO mice were compared using the comparative CT method (2−ΔΔCT = [(C_T gene of interest – C_T internal control) sample – (C_T gene of interest – C_T internal control) sample B]).

Transcriptome analysis

In order to determine the changes in the transcriptome after Wwc2 ablation, RNA from three wildtype, three heterozygous, and four homozygous Wwc2 KO embryos at E11.5 was isolated and analyzed by RNAseq analysis. Library preparation of the total RNA was performed with the NEBNext Ultra RNA directional Kit and paired read sequencing was performed using a NextSeq® 500 System with a read length of 1 × 80 bp. Using a molecular barcode, the samples were demultiplexed (bcl2fastq2) to fastq data and quality controlled (FastQC). Trimmmomatic was used for adapter trimming and read filtering. The resulting reads were aligned to the mouse reference genome (mm10) using Hisat2 and the aligned reads were sorted using samtools. The sorted and aligned reads were counted into genes using htsec-counts. The test for differential expression were performed using the r-package deseq2.

Tissue extraction preparation and western blot analysis

Tissue extraction from embryos was performed as described earlier. In brief, tissues were lysed in ice-cold lysis buffer (20 mM Tris-HCl pH 7.4, 20 mM NaCl, 1 mM EDTA, 50 mM NaF, 10 mM Na3P2O7, 1% Triton-X-100) containing protease (Roche) and phosphatase (Sigma) inhibitors. After the lysates were centrifuged at 14,000 × g for 45 min, supernatants were removed and stored at −80°C until further use. Western Blot analysis was performed using standard protocols. Antibodies used are listed in Supplementary Tab. 2.

Statistical analysis

For statistical analysis, the software GraphPad Prism 5 was used to perform a paired two-tailed Student’s t test. Data describing vascular networks were evaluated using GraphPad Prism 7 (Kruskal–Wallis test and Dunn’s multiple comparisons test) and AngioTool.

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research. AH, GW, P.I.N., CE, VH, V.C.B., JB, MM, PH, and JK analyzed data. AH, GW, P.I.N., JB, M.P.K., VCB, CE, PH, HP, and JK wrote the paper.

Conflict of interest
The authors declare that they have no conflict of interest.

Ethical approval
Animal experiments were performed in accordance with national guidelines and were approved by the local authorities of North Rhine-Westphalia, Germany.

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