Supplementary materials and methods

Mice

We generated a *miR-127* deleted construct with the neomycin resistance gene (Fig. S1A). Three large genomic DNA fragments were amplified by PCR using KOD HiFi DNA polymerase (Novagen) (Fragment A: chr12:109,590,618-109,595,291, Fragment B: chr12:109,595,397-109,597,519, Fragment C: chr12:109,597,500-109,599,896 respectively, from the mouse reference genome mm9 assembly (Fig. S1A). These fragments were independently cloned into the TOPO cloning vector (Invitrogen). *miR-127* was removed from fragment A by *Ava*I digestion (chr12:109,592,803-109,592,936). Cloned fragments were combined into the neoflox8 vector (provided by U. Lichtenberg, Cologne) including the neomycin resistance gene. The small gap between fragment A and fragment B was filled with a small PCR fragment. After G418 selection followed by transfection of the *miR-127* targeting construct into female 129Sv ES cells, the recombinants were screened by PCR and Southern blotting (Fig. S1B-D). The primers for screening are (short arm) neof1077F 5'-CGCCAATGACAAGACGCTGG and pEX1R 5'-CGTCTGCATGACCTAGAGGC. Primers for screening the miR127 deletion are Rtl1 9486R 5'-ACCTGGCCGACGTGTTTA and AW060F 5'-CCGAACGATGCTCTCCAAGTG. Targeted ES Cells were treated with *Cre* recombinase to delete the neomycin resistance gene (Fig. S1E). The primers for screening the loxP deletion are Rtl1 13940 5'-ATTTGCAGCAATCCGATTTT and Rtl1 14143 5'-TGTCTGTGTATGTGAATATGTGTGC. ES targeting and blastocyst injection was carried out by the Gene Targeting Facility at The Babraham Institute. Mice were crossed into and maintained on a C57BL/6J genetic background unless otherwise indicated. For allelic expression analysis, hybrid conceptuses were

made from Δ*miR-127* female mated with WT congenic male carrying *Dlk1-Dio3* cluster from *Mus musculus molossinus*. Experiments involving mice were carried out in accordance with the UK Government Home Office licensing procedures (licence 80/2567).

Placental histology

Dissected placentae were cut mid-sagittally and one half was fixed in 4% paraformaldehyde in 70 mM phosphate buffer and the other half fixed in 4% glutaraldehyde in Pipes buffer. Following fixation, the paraformadehyde-fixed half was dehydrated, embedded in paraffin wax, sectioned at 7µm and stained with haematoxylin and eosin. The glutaraldehyde-fixed half was dehydrated and embedded in Spurr epoxy resin (Taab, Aldermaston, UK) and a 1 µm vertical section cut close to the placental midline was stained with Toluidine Blue. Paraffin and resin embedded sections were analyzed using superimposed grids and systematic sampling within random fields and the Computer Assisted Stereological Toolbox (CAST v2.0). The proportion of placental compartments; the labyrinthine zone, junctional zone and decidua were determined by point counting on the haematoxylin and eosin sections and then converted into estimated volumes by multiplying their proportion by total placental weight. Labyrinthine morphometric analyses were performed on resin sections as described by Coan et al., 2004.

Rapid Amplification of cDNA Ends (5'RACE)

5'RACE was performed using First Choice® RLM-RACE (Ambion) using 10 µg of total RNA from E11 embryo and E15.5 placenta. Standard reactions were performed following the manufacturer's protocol. For first round synthesis each 25 µl reaction

contained 1X PCR buffer (KOD Hot Start, Novagen), 300 µM dNTPs, 1 mM $MgSO_4$, 0.5 U Hot Start KOD polymerase, 0.4 µM of each primer (5'RACE Outer primer and 11545F 5'-CAGTGGGCAGCTCTTGCATTTCCTG) and 1-2 µl RT reactions. The PCR cycling program was: 94ºC for 3 minutes, then 35 cycles at 94ºC for 30 seconds, 58ºC for 30 seconds, 72ºC for 2 minutes and a 7 minute extension cycle at 72ºC. The second round of PCR was performed as above using 1-2 µl of the first round PCR as the template. In this reaction the 5'RACE inner primer and RRACE1 5'-TGTCGTCGGTTGGAAAGGAGTGTGC were used. The products were cloned into pGEM-T easy (Promega) and sent for Sanger sequencing.

Real time RT-PCR

Total RNA was extracted from whole embryos and placenta at E16.5 using TRI Reagent (Ambion). Reactions were run in 12.5 µl in the presence of 1× SYBR Green JumpStart *Taq* ReadyMix (Sigma Aldrich), 400 nM primers. PCR conditions were 95°C for 15 min followed by 40 cycles at 95°C for 15 seconds, 66°C for 30 seconds and 72°C for 5 seconds on a DNA Engine OPTICON2 (MJ Research). All reactions were performed in triplicate. The primers used were as follows: Forward primer Rtl1Ex1aF 5'-CAAGGACTCTCCCTCTCCAC, Rtl1Ex1bF 5'-AGGCACCCGAGCAGAGAG, RtlEx1cF 5'-GCTCAGAGGCAATCAAGGAG, Rtl1Ex1dF 5'-GAAGGCACT-ATTGCATCCTGA, Rtl1Ex1eF 5'-AGTTTGGCCAAGGAAGGATT, Reverse primer RRACE1 5'-TGTCGTCGGTTGGAAAGGAGTGTGC. For reference, Tbp F 5'-GGCCTCTCAGAAGCATCACTA, Tbp R 5'-AGGCCAAGCCCTGAGCATAA or Pecam1 F 5'-TCCCTGGGAGGTCGTCCAT, Pecam1 R 5'-GAACAAGGCAGCGGGGTTTA (Wang et al., 2005).

For direct comparison of *Rtl1* expression levels between the Δ*miR-127* and the maternally inherited multi-microRNA deletion (Sekita et al., 2008). cDNAs were synthesised from both mutant placentae using SuperScriptIII according to the method described previously (Sekita et al, 2008). Reactions were run in 10 µl in the presence of 1× SYBR Green I Master (Roche), 400 nM primers. PCR conditions were 95°C for 5 min followed by 45 cycles at 95°C for 10 seconds, 66°C for 10 seconds and 72°C for 10 seconds on LightCycler 480 II (Roche). The primers, Rtl1Ex1aF and RRACE1, Tbp F and Tbp R, were used for *Rtl1 Ex1a* and *Tbp* analysis, respectively.

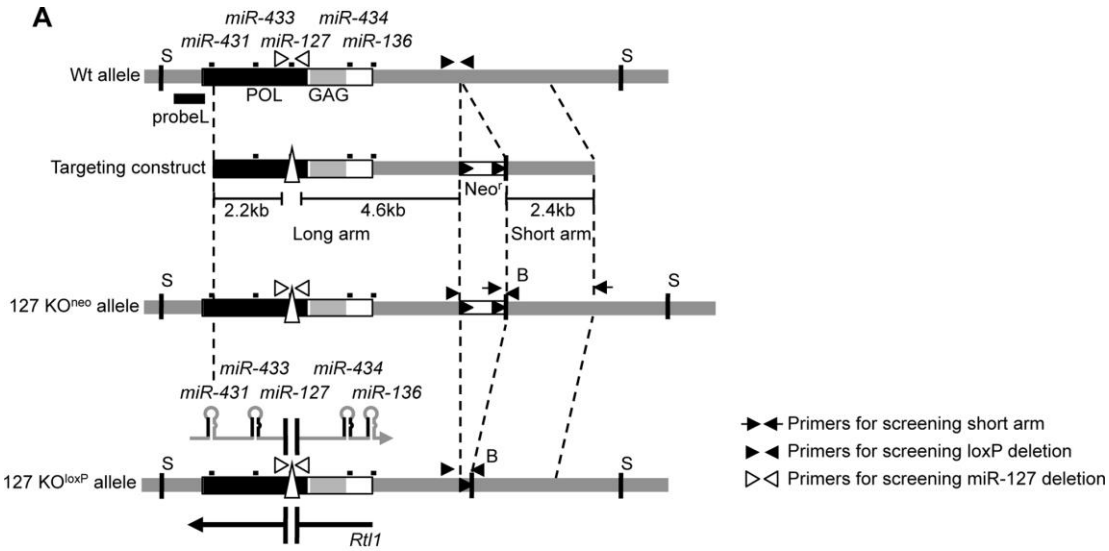
For allelic-specific expression analysis, five forward primers, Rtl1Ex1aF to Rtl1Ex1eF, and reverse primer RRACE8 5'-ACTTCTTGGAGTAGATTAGTGGGCAGCTC were used. The amplicons were gel purified using MinElute Gel Extraction Kit (Qiagen) and SNP (rs242380595) was detected by direct sequencing.

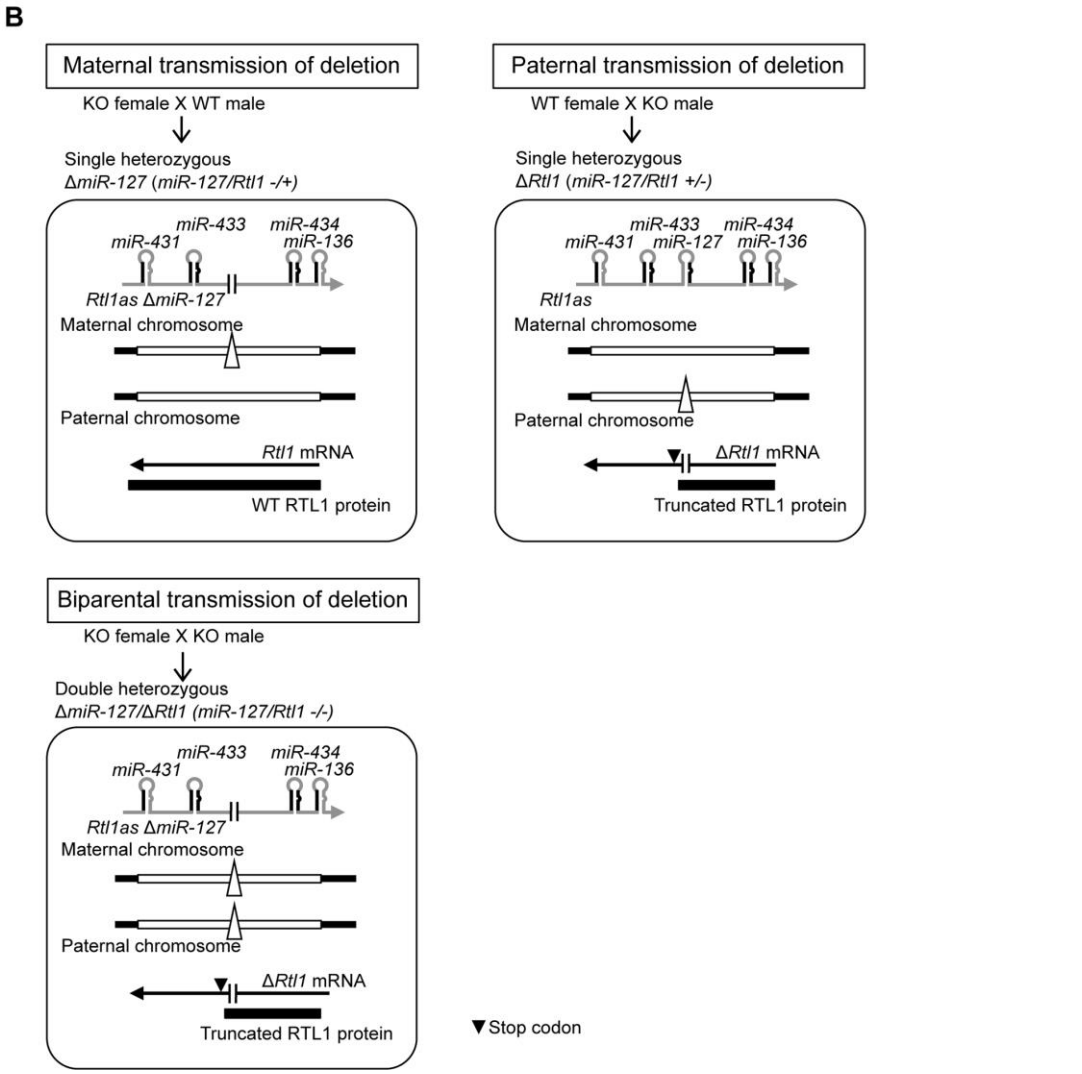
For mature miRNAs, we used TaqMan MicroRNA assays (Life technologies). *miR-127* (mmu-miR-127, 4427975-001183), *miR-431* (hsa-miR-431, 4427975-001979), *miR-433* (has-miR-433, 4427975-001028) *miR-434-3p* (mmu-miR-434-3p, 4427975-001140) and *miR-136* (mmu-miR-136, 4427975-002511), accumulation was monitored and *snoRNA202* (snoRNA202, catalog number; 4427975-001232) was used as standard internal control. First strand cDNA was synthesized from total RNA from embryo and placenta using RevertAid H minus First Strand cDNA synthesis kit (Fermentas). Briefly, multiplex reverse transcription was carried out with 10ng total RNA, 0.5mM dNTPs, 120unit M-MuLV reverse transcriptase, four TaqMan miRNA RT primers mix (0.65 µl each) and 1 x RT buffer in 6 µl reaction mix with 10 µl mineral oil overlay. The reverse transcription reaction consisted of 30 min at 16˚C, 30 min at 42˚C and 5 min at 85˚C. Real time PCR was carried out using TaqMan

Universal Master Mix, No AmpErase UNG (Applied Biosystems) following the manufacture's instructions. Signals were detected using the ABI Fast real time PCR Systems (Applied Biosystems) and quantification carried out by the comparative Ct method. Experiments were repeated in triplicate.

Western blotting

Proteins were isolated from E16.5 embryos and placentae. Ten micrograms of protein were separated by 6% SDS-PAGE and then blotted to PVDF membranes. After incubation with 5% skimmed milk in 0.1% PBST for 60 min, the membrane was washed three times with 0.1% PBST and incubated with antibodies against RTL1 (1:1000) or α-Tublin (1:10000, Sigma Aldrich, T6199). Membranes were washed three times for 10 min and incubated with a 1:2000 dilution of HRP conjugated anti-rabbit or anti-mouse antibodies for 2h. Blots were washed with 0.1% PBST three times and developed with the ECL system (Amersham Biosciences) according to the manufacturer's protocol. The anti-RTL1 antibody (YZ2844) was produced by YenZym Antibodies, LLC (South San Francisco, CA). The RTL1 antigen (chr12:109,594,459-109,595,139) and His fusion protein was immunised in rabbits and the antibody was purified.


A
miR-433 miR-434
miR-431 miR-127 miR-136
Wt allele
S
POL
GAG
probeL
Targeting construct
Neo^r
2.2kb
4.6kb
2.4kb
Long arm
Short arm
B
127 KO^neo allele
127 KO^loxP allele
Rtl1
Primers for screening short arm
Primers for screening loxP deletion
Primers for screening miR-127 deletion


B
Maternal transmission of deletion
KO female X WT male
Single heterozygous
ΔmiR-127 (miR-127/Rtl1 -/+)
miR-433 miR-434
miR-431 miR-136
Rtl1as ΔmiR-127
Maternal chromosome
Paternal chromosome
Rtl1 mRNA
WT RTL1 protein
Paternal transmission of deletion
WT female X KO male
Single heterozygous
ΔRtl1 (miR-127/Rtl1 +/-)
miR-433 miR-434
miR-431 miR-127 miR-136
Rtl1as
Maternal chromosome
Paternal chromosome
ΔRtl1 mRNA
Truncated RTL1 protein
Biparental transmission of deletion
KO female X KO male
Double heterozygous
ΔmiR-127/ΔRtl1 (miR-127/Rtl1 -/-)
miR-433 miR-434
miR-431 miR-136
Rtl1as ΔmiR-127
Maternal chromosome
Paternal chromosome
ΔRtl1 mRNA
Truncated RTL1 protein
▼Stop codon

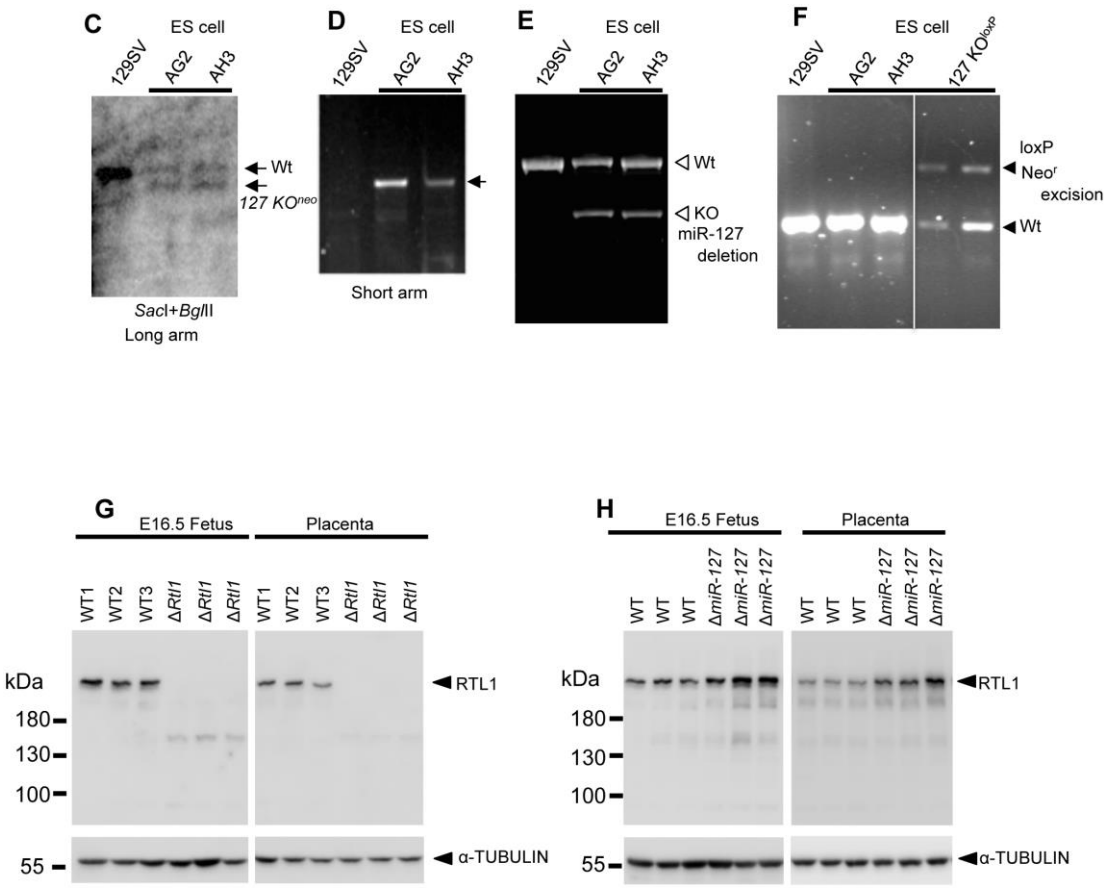


Fig. S1. Production of Δ*Rtl1* and Δ*miR-127* mice. (A) Schematic representation of the knockout strategy. The third exon of *Rtl1* is shown as an open box. Gag homology domains are shaded grey and Pol homology domains are black. LoxP sites are shown as solid triangles and the deleted site is indicated as an open triangle. *Sac*I [S] and *Bgl*II [B] restriction sites and probeL were used for recombinant screening the long arm of the targeted allele by Southern blotting. Three primer sets were used for PCR screening of the targeted allele. Arrowheads show primers for screening the short arm of the targeted allele, 127 KO^{neo}. The neomycin-resistance gene (*Neo^r*) was removed in ES cells by *Cre recombinase.* The solid arrow heads indicate primers for screening of the Neo^r excluded allele (*127 KO^{loxP}*). The open arrows are primers for screening for the *miR-127* deleted allele. (B) Single heterozygous and double heterozygous are indicated. The upper-left panel shows maternal transmission of the

small deletion. These single heterozygous mutant mice, *miR-127/Rtl1 -/+* lose *miR-127* expression. The upper-right panel shows reciprocal paternal transmission of the same deletion. Here, the small deletion makes *Rtl1* mRNA without the *miR-127* binding site. The deletion also induces a frame shift resulting in a nonsense mutation in the *Rtl1* ORF which would result in a truncated RTL1 protein. Hence, the single heterozygous mutant mice, *miR-127/Rtl1 +/-* lose functional RTL1 protein. The lower panel shows biparental transmission of the small deletion. These double heterozygous mutant mice, *miR-127/Rtl1 -/-* lose *miR-127* expression and functional RTL1 protein expression. (C) Southern blots of genomic DNA for screening for long arm recombination. Southern blots of genomic DNA from ES cells that have WT alleles (left; 129Sv), and *127 KO*^{neo} allele (middle; AG2 and right; AH3). (D) PCR screening for the short arm of the *127 KO*^{neo} allele. The left lane shows WT (no signal) and the middle and right lanes indicate *127 KO*^{neo} alleles. (E) PCR results show the *miR-127* deleted allele (lower signal) and WT allele (upper signal) in 129Sv, AG2 and AH3 ES cells. (F) After *Cre recombinase* treatment, PCR shows the WT allele (lower signal) and the *Neo*^{r} deleted allele (upper signal). The *Neo*^{r} inserted allele is not detected by PCR. (G) Western blotting shows that Δ*Rtl1* embryos and placentae lacked RTL1 protein expression. The premature truncated RTL1 protein cannot be observed in predicted size (95.6 kDa) in both Δ*Rtl1* embryos and placentae. (H) Western blotting shows Δ*miR-127* embryos and placentae with increased RTL1 protein expression.

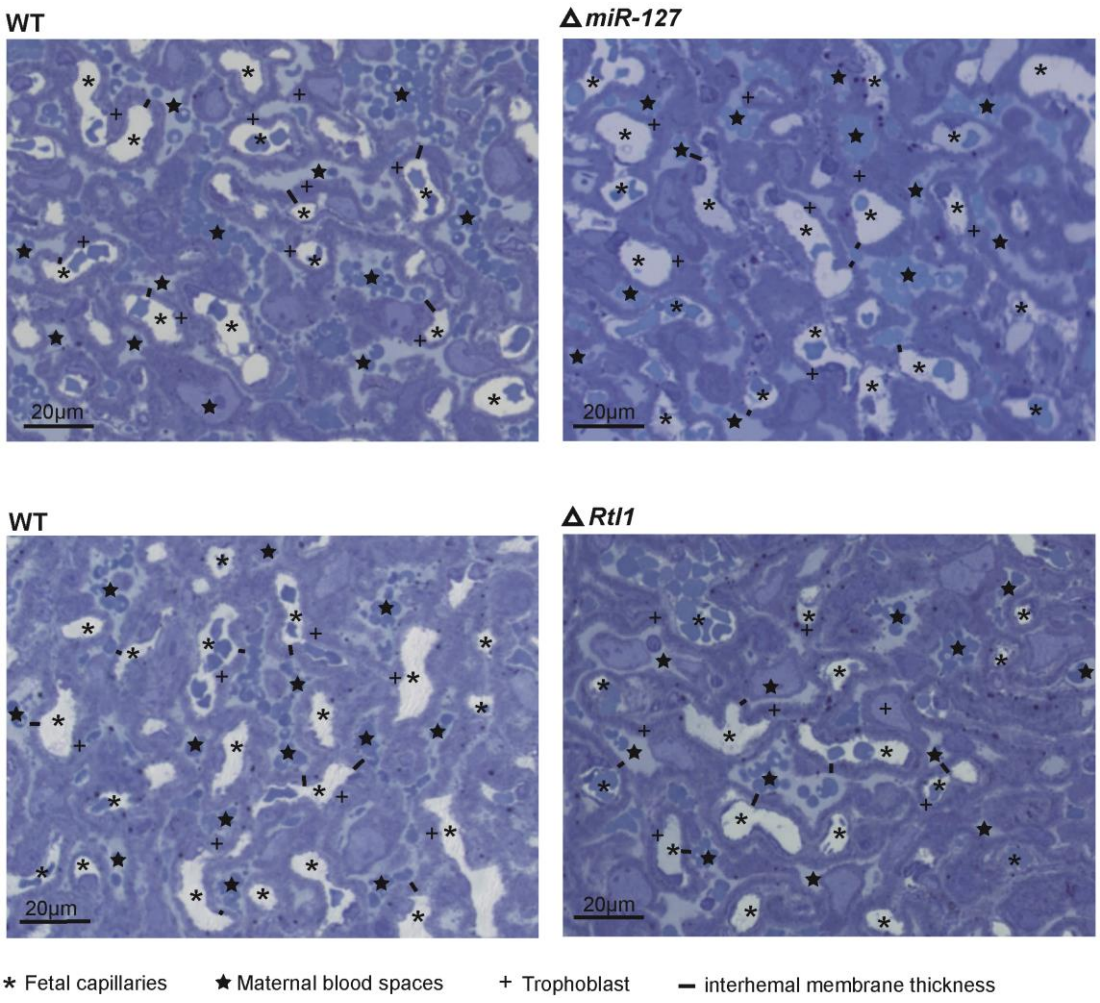
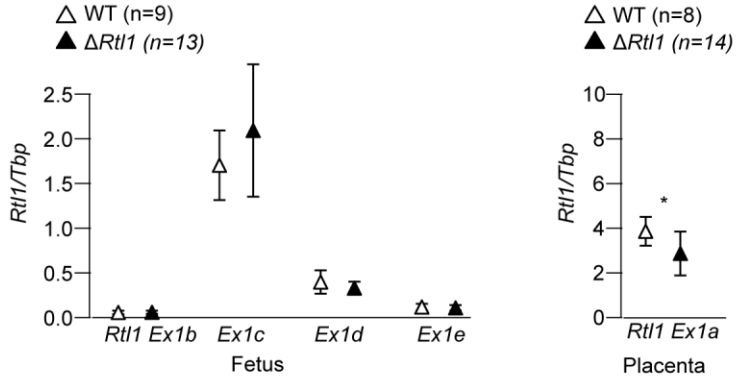


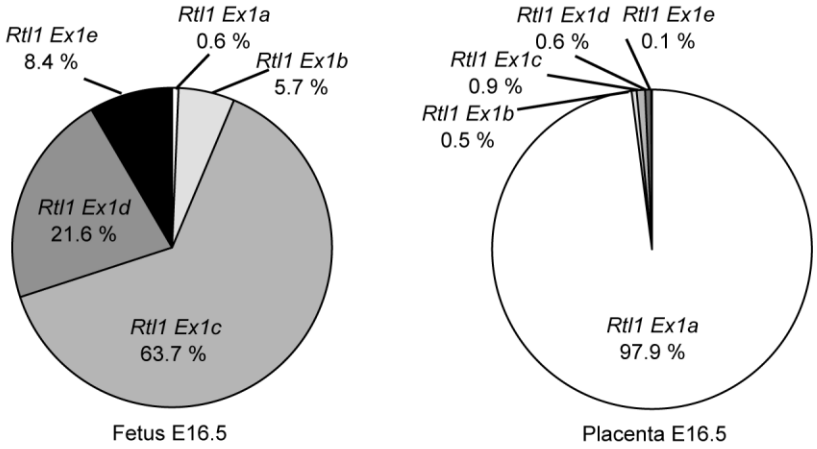
Fig. S2. Photomicrographs of placental labyrinthine zone. The photomicrographs show toluidine stained resin-embedded sections of E18.5 placentae. Fetal capillaries, trophoblast and maternal blood spaces from which the volume fractions, absolute volumes and surface areas and fetal capillary length can be derived as well as the interhemal membrane thickness (black bar) determined, using established, published stereological methods (Fig. 2E,F and table S3).

A

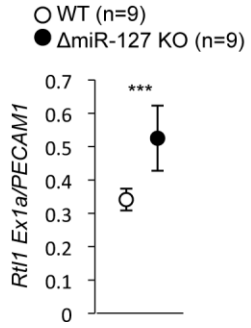


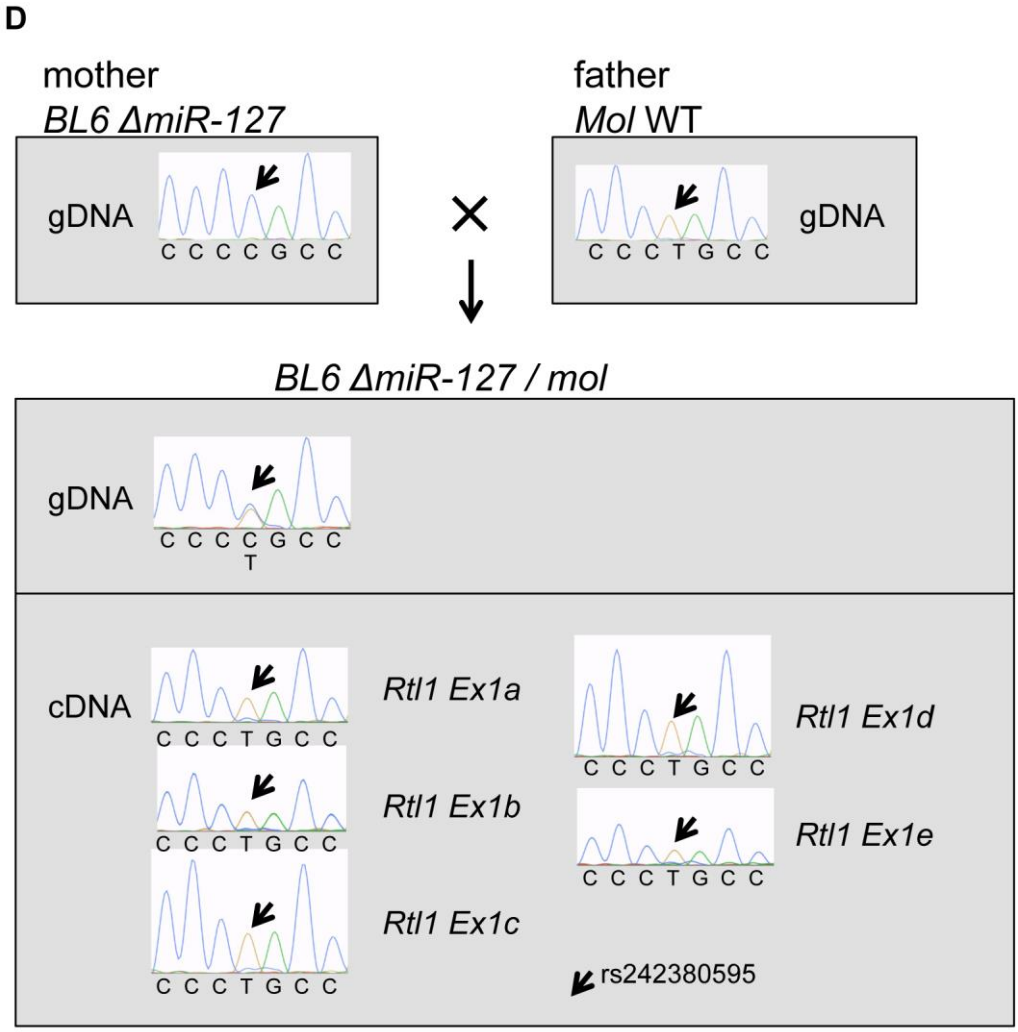
Student's t-test was used for statistic analysis . *** P<0.005

B



C





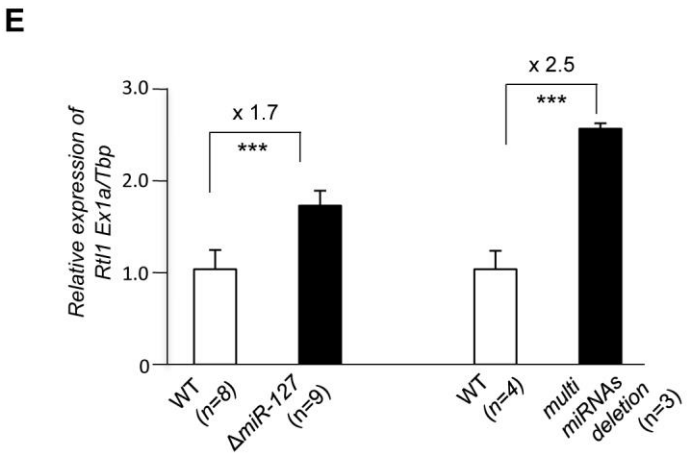


Fig. S3. Expression analysis for each alternative *Rtl1* transcript and the miRNAs. (A) As expected, the deleted *Rtl1* mRNAs were transcribed and stable in Δ*Rtl1*. There is no significant difference in *Rtl1* mRNAs between Δ*Rtl1* and WT embryos. In placenta, *Rtl1 Ex1a* expression was reduced slightly compared to WT.

Error bars represent s.d. Statistically significant differences are indicated by an asterisk (student's t-test; *; P<0.05). (B) Graphs indicate transcription level of each alternative transcript at E16.5 fetus and placenta. (C) Since the expression of *Rtl1 Ex1a* normalised to the endothelial cell marker *PECAM1/CD31* increased *in* Δ*miR-127* placenta, the expansion of endothelial cells does not cause the increase of *Rtl1 Ex1a.* (D) All five *Rtl1* alternative transcripts are exclusively transcribed from the paternal chromosome in Δ*miR-127*. (E) Quantitative expression analysis for the *Rtl1 Ex1a* transcript in Δ*miR-127* and the multi-miRNA deletion placentas (Sekita et al., 2008) at E16.5.

Fig. S4. The sequences of alternative *Rtl1* transcripts. The sequences of 5' RACE products are shown. Each box indicates alternative exon1 or exon2. Unboxed sequence represents the 5' end of exon3. *Rtl1 Ex1c* is reported in GeneBank database as EU434918. *Rtl1 Ex1a* was cloned from E15.5 placenta. The other three were cloned from E11 embryos.

Table S1 The lethality of the Δ*miR-127 and* Δ*Rtl1* mice.

Maternal Δ*miR-127*	WT	KO
N3	44	32
N4	24	23
N5	21	20
N6	78	82
N7	77	56
N8	51	51
N9	53	45
N10	19	15
>N11	150	154

The number of WT and Δ*miR-127* mice which grew to adulthood generation are shown. The Δ*miR-127* mice and WT mice have comparable survivability during backcrossing with C57BL/6J.

Paternal Δ*Rtl1*	WT	KO
N2	26	29
N4	8	8
N5	36	8*
N6	30	7*
N7	93	47*
N9	57	4*
N10	15	2*
N12	56	4*
N14	40	10*

The number of WT and Δ*Rtl1* mice which grew to adulthood generation are shown. The survivability of Δ*Rtl1* mice decreased during backcrossing with C57BL/6J. Pearson's chi-square test was used for statistic analysis (*; P<0.05)

Paternal Δ*Rtl1* (129aa X C57BL/6J hybrid background)

	WT	KO
N14	38	38

The number of WT and Δ*Rtl1* mice in 129aa X C57BL/6J hybrid background are shown. The 129aa background can rescue the lethality of the Δ*Rtl1* mice in N14 generation.

Supplemental Table S2 The survivability of Δ*miR-127 and* Δ*Rtl1* at embryonic stage.

Maternal Δ*miR-127*		KO/WT weight Ratio (%)	
Stage	WT:Δ*miR-127*	embryo	placenta
E12.5	9: 5	105.4	97.5
E14.5	16:21	102.3	101.1
E16.5	40:32	99.0	111.6*
E18.5	22:19	102.0	118.5*
Total	87:77		

Paternal Δ*Rtl1*		KO/WT weight Ratio (%)	
Stage	WT:Δ*Rtl1*	Fetus	placenta
E12.5	2: 1	82.7	106.1
E14.5	19:26	101.1	81.6*
E16.5	12:20	80.7*	82.2*
E18.5	31:21	79.5*	77.5*
Total	64:68		

Student's t-test was used for Statistic analysis * $p<0.05$

Supplemental Table S3. Stereological analysis of placentae from *miR-127* and *Rtl1* single or double homozygous knockout mice on E18.5.

	WT (N=4)	Δ*miR-127*/+ (N=3)	Δ*miR-127*/WT (%)	WT (N=6)	+/Δ*Rtl1* (N=4)	Δ*Rtl1*/WT (%)	WT (N=4)	Δ*miR-127*/ Δ*Rtl1* (N=7)	KO/WT (%)
Placental compartment volume; mm^3 (proportion; %)									
Lz	46.3±5.7 (51.7)	65.9±5.1** (57.7)	142.3	46.4±4.3 (55.3)	30.0±9.4*** (41.1)*	64.7	41.1±4.1 (49.9)	28.5±5.4*** (45.0)*	69.3
Jz	24.5±9.2 (26.9)	20.9±8.1 (17.7)	85.3	20.2±4.9 (23.9)	22.6±4.1 (31.3)*	111.9	23.6±3.8 (28.6)	19.0±4.9 (29.8)	80.5
Db	13.6±2.6 (15.4)	10.2±6.0 (9.6)	75.0	12.2±2.3 (14.7)	10.7±1.4 (15.1)	87.7	9.7±0.8 (11.8)	10.2±2.4 (16.3)*	105.2
Ch	5.4±1.2 (6.0)	18.6±14.2 (15.1)	344.4	5.1±2.9 (6.1)	9.2±4.0 (12.5)*	180.4	8.2±3.5 (9.8)	5.8±2.9* (8.9)*	70.7
Labyrinthine compartment volume; mm^3 (proportion; %)									
MBS	7.4±0.8 (16.5)	9.9±2.4 (15.0)	133.8	6.2±2.3 (13.3)	4.0±1.1 (13.7)	64.5	10.8±2.0 (26.2)	6.4±1.8** (22.8)	59.3
FC	6.5±1.5 (15.3)	12.3±2.7* (18.6)	189.2	7.3±1.3 (15.7)	3.3±1.7** (10.7)**	45.2	6.4±0.9 (15.6)	3.7±2.1*** (12.5)	57.8
LT	32.3±4.1 (68.2)	43.7±5.6* (66.4)	135.3	32.9±2.5 (71.0)	22.7±7.1** (75.6)	69.0	23.9±3.2 (58.2)	18.3±3.0** (64.7)	76.6
Theoretical diffusion capacity; $mm^2.min^{-1}.kPa^{-1}$									
	12.8±3.5	19.3±3.5*	150.3	14.5±2.5	9.1±3.6*	62.6	14.6±2.6	9.4±3.8***	64.2
Specific diffusion capacity; $mm^2.min^{-1}.kPa^{-1}.g$ fetus									
	11.6±2.7	16.8±1.0*	145.5	11.4±1.8	8.8±3.5	77.5	11.7±2.5	9.2±1.9**	78.1
Lz interhemal membrane surface areas; cm^2									
MBS	23.6±7.3	35.5±6.8*	150.4	35.6±3.9	23.8±9.6*	66.8	31.9±5.5	21.2±4.3***	66.5
FC	21.4±3.6	33.1±7.7*	154.7	21.0±3.9	14.3±4.7*	68.3	17.4±2.8	11.4±3.6***	65.4
mean	22.5±5.8	34.3±5.9*	152.4	28.3±3.7	19.0±7.1*	67.4	24.7±3.3	16.3±3.8***	66.1
Interhemal membrane harmonic mean thickness; µm									
T_h	3.2±0.1	3.2±0.1	100.3	3.4±0.4	3.6±0.1	106.7	2.9±0.3	3.2±0.6	107.0
Labyrinthine fetal capillaries									
Length (m)	112.0±21.4	184.2±11.9***	164.4	136.0±20.5	85.2±31.2**	62.6	113.1±32.1	79.6±15.3*	70.4
Diameter (µm)	9.2±0.7	8.2±1.0	90.0	7.5±1.4	8.4±1.0	111.9	11.2±0.9	10.2±1.6	91.1

Data are presented as mean±SD. Student's t-test was used for Statistic analysis * P>0.05, ** P>0.01, *** P>0.005.
Abbreviations: Lz, labyrinthine zone; Jz, junctional zone; Db, decidua basalis; Ch, chorion; MBS, maternal blood spaces; FC, fetal capillaries; LT, labyrinthine trophoblast.