The *H19* lincRNA is a developmental reservoir of miR-675 that suppresses growth and *Igf1r*

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The *H19* large intergenic non-coding RNA (lincRNA) is one of the most highly abundant and conserved transcripts in mammalian development, being expressed in both embryonic and extra-embryonic cell lineages, yet its physiological function is unknown.

Here we show that miR-675, a microRNA (miRNA) embedded in *H19*'s first exon, is expressed exclusively in the placenta from the gestational time point when placental growth normally ceases, and placenatas that lack *H19* continue to grow. Overexpression of miR-675 in a range of embryonic and extra-embryonic cell lines results in their reduced proliferation; targets of the miRNA are upregulated in the *H19* null placenta, including the growth-promoting insulin-like growth factor 1 receptor (*Igf1r*) gene. Moreover, the excision of miR-675 from *H19* is dynamically regulated by the stress-response RNA-binding protein HuR. These results suggest that *H19*'s main physiological role is in limiting growth of the placenta before birth, by regulated processing of miR-675. The controlled release of miR-675 from *H19* may also allow rapid inhibition of cell proliferation in response to cellular stress or oncogenic signals.

The *H19* locus expresses high levels of a 2.5-kilobase (kb) RNA polymerase II-dependent transcript, which is spliced, capped, polyadenylated and exported into the cytoplasm¹⁻². Indeed, *H19* is the second most abundant transcript in placenta (Supplementary Fig. S6b) and levels are higher still in fetal liver (Fig. 1c). *H19* is imprinted with maternal expression, and has been implicated in tumour suppression, but its physiological function is unknown at present³⁻⁶. The *H19* transcript contains only short open reading frames, which are poorly conserved between mice and humans, and thus appears to be a non-coding RNA, one of the first lincRNAs to be discovered¹⁻³. *H19* is the primary miRNA (pri-miRNA) template for miR-675, we determined the expression profile of the miRNA. As *H19* derives its functionality from miR-675, we examined its expression in tissues where *H19* is highly transcribed. Unexpectedly, both species of miR-675 were barely detectable in fetal liver (endoderm-derived tissue) and fetal heart (mesoderm-derived tissue) at all stages of embryonic development tested, despite the vast

One way by which lincRNAs may acquire functionality is by acting as the precursor to small RNAs capable of regulatory function, such as microRNAs (refs 9–11). Indeed, exon 1 of *H19* harbours a miRNA-containing hairpin that has been found to serve as the template for two distinct miRNAs, miR-675-5p and miR-675-3p (ref. 12), and it has been suggested that it may be these miRNAs that confer functionality on *H19* (refs 4,12,13; Fig. 1a). Moreover, the miR-675 stem loop was shown to be one of the most highly conserved features of the *H19* RNA during mammalian evolution, indicating that selective pressure may be higher on the miRNAs than on *H19* as a whole⁷.

**RESULTS**

**miR-675 is expressed in placenta but processing is strongly inhibited**

To investigate the possibility that *H19* drives its functionality from miR-675, we determined the expression profile of the miRNA. As *H19* is the primary miRNA (pri-miRNA) template for miR-675, we examined its expression in tissues where *H19* is highly transcribed. Unexpectedly, both species of miR-675 were barely detectable in fetal liver (endoderm-derived tissue) and fetal heart (mesoderm-derived tissue) at all stages of embryonic development tested, despite the vast

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Figure 1  miR-675 is expressed in the late gestation placenta but suppressed in the embryo. (a) Schematic representation of the H19 transcriptional unit with miR-675 shown in red embedded within H19 exon 1. The black arrow indicates the transcriptional start site. (b–e) Expression determined by qRT–PCR for miR-675-5p (blue), miR-675-3p (orange) and H19 (green) in fetal heart (b), liver (c), fetal brain (d) and placenta (e). Expression levels are expressed as the number of molecules per picogram of total RNA with H19 levels on the left y axis and miR-675 levels on the right. (b) n = 3. (c) n = 3 for all E13.5 and all H19 experiments, and n = 4 for all others. (d) n = 3. (e) n = 4 for all miRNA experiments; n = 3 for E11.5, E13.5, E15.5 and E17.5 values for H19 experiments; and n = 6 for the E19.5 H19 experiment. Error bars indicate the s.e.m.

Figure 2  HuR binds to full-length H19 and inhibits processing of miR-675. (a) Venn diagram indicating the numbers of proteins that were identified by RNA affinity assay (see Methods) as binding to H19 in the region of the miR-675 stem loop (Stem) and to control segments including H19 upstream of the stem loop (5' H19) and of the Kcnq1ot1 RNA (Kcnq1ot1). (b) Microarray data from a published study of transcription during placentation development19 were reanalysed and the kinetics of HuR expression are shown from E8.5 until birth (P0). Note that HuR expression is inverse to that of miR-675 during placentation development. Data are from at least two biological and two technical replicates. (c) RNA immunoprecipitation with an antibody against HuR indicates binding to H19 in the placenta at a gestational time point when miR-675 is suppressed (E11.5) but not when it is expressed (E19.5). The enrichment of RNA over a random IgG control is shown following normalization to the 18s RNA. Levels of Actb and Gapdh are included as positive and negative controls, respectively. n = 9. (d) RNA immunoprecipitation with an antibody against HuR indicates binding to H19 in MEF cells. The enrichment of RNA over a random IgG control is shown following normalization to the 18s RNA. Levels of p21 and Gapdh are included as positive and negative controls, respectively. n = 11. (e) The expression of miR-675-5p, miR-675-3p or miR-16 in MEF cells following treatment with either an siRNA against HuR (si-HuR) or a non-targeting scrambled control (si-scrambled) as determined by qRT–PCR. n = 4. (f) The expression of miR-675-5p, miR-675-3p or miR-16 in MEF cells that genetically lack HuR (HuR−/−). n = 5. (g) The expression of miR-675-5p, miR-675-3p or miR-16 in C2C12 cells following treatment with either an siRNA against HuR (si-HuR) or a non-targeting scrambled control (si-scrambled) as determined by qRT–PCR. All error bars indicate the s.e.m. P values were determined by Student’s t-test. *P < 0.05, **P < 0.01, ***P < 0.001.
miR-675 processing is inhibited by the RNA-binding protein HuR

As inhibition seems to be a dynamic process in the placenta, we reasoned that RNA-binding proteins might contribute to miR-675 regulation and hence performed RNA affinity assays followed by mass spectrometry to identify proteins that bind H19 in the region of the miR-675 stem loop (see Methods). We used partially differentiated trophoblast stem (TS) cell lysate as a cell culture approximation of early gestation placenta as these cells do not express miR-675 despite robust H19 levels (Supplementary Fig. S1b,c). Using this approach we identified 49 proteins that were bound selectively to H19 in the region of the miR-675 stem loop (Fig. 2a). Notably, these included a number of proteins that are implicated in RNA metabolism and small RNA regulation, such as Upf1, Zc3ch11, Luc7l and HuR. Amongst these, the RNA-binding protein HuR stood out as a candidate for regulating miR-675 processing, for a number of reasons: first, there is a putative HuR-binding site in H19 55 base pairs (bp) upstream from the miR-675 stem loop (AUUUUA), second, expression of HuR in the placenta declines during development as that of miR-675 increases\(^*\) (Fig. 2b), and third, HuR is known to afford messenger RNAs protection from endonucleases (the miRNA-processing enzymes Drosha and Dicer are both RNAse III class endonucleases\(^*\)). Finally, knockout of HuR results in specific defects of the placenta\(^{21}\). We also found HuR expression to be higher in the liver, where miR-675 is silent, than it is in the late gestation placenta (data not shown).

We performed RNA immunoprecipitation with an antibody to HuR and found that it bound to full-length H19 in placenta at E11.5, when miR-675 is suppressed and HuR levels are high, but not at E19.5, when miR-675 is expressed and HuR levels are lower (Fig. 2c). We sought to identify a cell culture model in which to perform functional studies. miR-675 is suppressed in primary mouse embryonic fibroblast (MEF) cells despite robust expression of H19 (Supplementary Fig. S1d), suggesting that inhibition of processing occurs in these cells; RNA immunoprecipitation experiments indeed confirmed HuR binding to H19 (Fig. 2d). To assess the role of HuR in the suppression of miR-675 processing, we performed short interfering RNA (siRNA)-mediated knockdown in MEF cells and found that levels of both miR-675-5p and miR-675-3p were increased approximately 2- and 1.5-fold, respectively, in the absence of HuR (Fig. 2e and Supplementary Fig. S2a,b). A similar effect was observed in MEF cells genetically deficient for HuR (HuR\(^{-/-}\)), where both miR-675-5p and miR-675-3p were increased by approximately 2- and 2.2-fold respectively relative to wild-type controls (Fig. 2f). Finally, we ablated HuR in a myoblast cell line (C2C12) by siRNA knockdown and again found that miR-675-5p and miR-675-3p
levels were increased in the absence of HuR, by 2.8- and 3.5-fold, respectively (Fig. 2g and Supplementary Fig. S2c,d). In all cell culture models of HuR deficiency, levels of miR-16 were unchanged relative to controls, suggesting that the regulatory effect of HuR ablation is specific to miR-675 as opposed to the broader miRNA-processing pathway. Thus, HuR negatively regulates processing of both species of miR-675. Luc7l knockdown also resulted in increased miR-675 levels, suggesting that HuR is not the only negative regulator of processing (Supplementary Fig. S2b).

**HuR does not block miR-675 processing at the Dicer step and probably blocks Drosha**

We next sought to identify where in the miR-675-processing pathway HuR acts to inhibit processing. miRNAs are processed sequentially from longer hairpin-containing RNAs, first in the nucleus from the pri-miRNA by Drosha to create the pre-miRNA and second from longer hairpin-containing RNAs, first in the nucleus from the pri-miRNA by Drosha to create the pre-miRNA and second in the cytoplasm by Dicer to create the mature miRNA (ref. 22). The abundance of H19 (the pri-miRNA) suggests that processing of miR-675 is inhibited at the Drosha stage. To investigate this further, we performed northern blot experiments on fetal liver and placental RNA and found only low levels of pre-miR-675, the product of Drosha cleavage of H19 (Fig. 3a,b). Moreover, when we transfected synthetic pre-miR-675 into MEF cells, we observed an increase in mature miR-675 and this increase was not altered by the presence or absence of HuR, showing that processing by Dicer was not affected by HuR (Fig. 3c). Finally, our RNA immunoprecipitation experiments showed that HuR binds to the full-length H19 RNA (Fig. 2c,d). Taken together these data suggest that HuR binds the full-length H19 RNA and inhibits processing of miR-675 at the Drosha step. Nevertheless, HuR ablation does not result in complete processing of H19, suggesting that other mechanisms exist to protect H19 from Drosha cleavage, possibly including the other H19 RNA-binding proteins we identified. Drosha localizes exclusively to the nucleus whereas the H19 RNA is known to localize predominantly to the cytoplasm1,2, and thus it is likely that HuR (and perhaps other RNA-binding proteins) protects H19 from processing by Drosha while it is localized to the nucleus, but that another level of protection is afforded by nuclear export of H19. A family of Igf2 mRNA-binding proteins have been implicated in the cytoplasmic localization of H19 (ref. 23) and these proteins are thus additional candidates for conferring inhibition of miR-675 processing. To determine whether HuR affects the nuclear export of H19, we performed nuclear and cytoplasmic RNA fractionations from wild-type and HuR−/− MEF cells and found no change in the abundance of nuclear H19 (Fig. 3d,e). Moreover, fractionation of placental RNA revealed that the increasing miR-675 expression observed in that organ is not due to increased nuclear H19 levels (Fig. 3f–i).

**miR-675 slows cell proliferation**

The fact that miR-675 has been conserved during evolution, despite suppression of its processing, suggests a function for the miRNA as well as a need for precise regulation of its dosage. To examine its function we transiently transfected miR-675-5p and miR-675-3p mimics into MEF, C2C12, TS and ES cell lines and found that the proliferation rate of all cell lines was reduced by at least 50% in the presence of miR-675 when compared with a scrambled control (Fig. 4a–d). TdT-mediated dUTP nick end labelling (TUNEL) staining showed
that this effect was not due to increased apoptosis (Supplementary Fig. S3a), rather we noticed that the negative regulator of cell cycle Rb1 was upregulated by miR-675, indicating a possible reduction of cell cycle rate (Supplementary Fig. S3b). Interestingly, it was not a single species of miR-675 that was responsible for the reduced proliferation, but rather miR-675-5p slowed the rate of proliferation of ES, TS and to a lesser extent MEF cells, whereas miR-675-3p slowed that of MEF and C2C12 cells. To confirm these results we used the A2lox.cre ES cell line24 to create cells capable of doxycycline-induced expression of either miR-675-5p (A2lox-5p), miR-675-3p (A2lox-3p) or a scrambled control (A2lox-scrambled). To avoid inhibition of processing we placed these miRNAs in the miR-30 hairpin context and observed selective upregulation of either species of miR-675 on the addition of doxycycline (Supplementary Fig. S4). Cell proliferation assays confirmed the results of the transient miR-675 mimic experiments in ES cells, in that proliferation of the A2lox-5p cell line was slowed by the addition of doxycycline, whereas no effect was observed on the A2lox-3p or A2lox-scrambled cell lines (Fig. 4e–g).

H19 was first shown to have tumour-suppressor properties by overexpression in the G401 metastatic rhabdoid tumour cell line5. We investigated whether miR-675, rather than H19 itself, may have been responsible for this previously reported effect by overexpressing the smaller RNA in these cells. Indeed, we observed reduced proliferation of G401 cells when treated with miR-675-5p as opposed to a scrambled control (Fig. 4h). Finally, we took advantage of the fact that both H19 and miR-675 become expressed in differentiating C2C12 cells (Supplementary Fig. S5) to perform antagomiR-mediated loss-of-function experiments and found that proliferation of these cells increased when miR-675-3p was inhibited (Fig. 4i). Taken together these results show that miR-675 is a functional component of the H19 RNA, capable of suppressing cell proliferation.

Deletion of both H19 and miR-675 results in placental overgrowth

Given the growth-limiting effect of miR-675, it was interesting to note that expression of the miRNA in the placenta is concomitant with the natural cessation of the growth of that organ32. Moreover, mice that carry a 13-kb deletion that includes H19, miR-675 and 10 kb of regulatory sequences upstream of H19 show an overgrowth phenotype that is more severe in the placenta (145% wild-type) than the embryo36–38 (123% wild type). The interpretation of this phenotype, however, is complicated by the fact that the deletion (of the imprinting control region upstream of H19) also results in overexpression of the linked Igf2 gene28. To examine the possibility that the H19 RNA itself regulates placental growth, we studied an alternative H19 mouse model (H19Δ3) that carries a 3-kb deletion of just the H19 transcription unit, including miR-675 (ref. 30). This deletion specifically ablates the H19 RNA without affecting expression of Igf2 in the placenta39 (Supplementary Fig. S6a). Notably, placentas carrying the H19Δ3 allele were 32% larger than wild types at E18.5, whereas the mutant embryos themselves were only 8% larger than wild type, in agreement with previous results50 (Fig. 5a and Supplementary Fig. S6c). Expression of miR-675 in the placenta correlates with a rapid drop in the expression of HuR and the natural cessation of placental growth, which does not occur in the absence of H19 and miR-675 (Fig. 5b), implying that miR-675 is a negative regulator of placental size.

Figure 5 The phenotype and transcriptome of the H19Δ3 placenta imply that miR-675 is a negative growth regulator in this tissue. (a) Placental weight was measured for homozygous H19Δ3 crosses and compared with the wild type, revealing an overgrowth phenotype for the knockout that is more severe in the placenta than the embryo. The number of placentas measured for each data point is indicated above each bar. Error bars indicate the s.e.m. P values were determined by Student’s t-test. ** P < 0.001. (b) Representation based on real data showing the correlation of mir-675 (blue) and HuR (ref. 19; orange) expression in the placenta with the developmental weight of that organ (black). The weight of the H19Δ3 placenta is also indicated (dashed line). At E13.5 there is a rapid drop in HuR expression concomitant with the induction of mir-675. At approximately E15.5, the wild-type placenta ceases to increase in mass41; however, this does not occur in the H19Δ3 placenta. (c) Enriched gene ontology terms in RNA sequencing data from the labyrinthine layer of a day E18.5 wild-type and H19Δ3 placenta. Categories for genes upregulated (black) and downregulated (white) in the H19Δ3 placenta are shown.
**The H19Δ3 placental transcriptome reveals targets of miR-675**

miR-675 is expressed most highly in the labyrinthine zone of the placenta when compared with the junctional zone (Supplementary Fig. S7a–d); to determine the effect of miR-675 on the H19Δ3 transcriptome, we performed RNA sequencing on RNA from the labyrinthine zone in the E18.5 H19Δ3 placenta. We found 285 genes upregulated and 39 downregulated by greater than twofold in the mutant versus the control labyrinth (Supplementary Fig. S7e). Gene ontology categories enriched in the H19Δ3 transcriptome were consistent with growth and morphogenesis of the placenta (Fig. 5c). H19 has previously been reported to regulate a network of imprinted genes in fetal muscle but not placenta4. Indeed, we observed no specific deregulation of imprinted genes in the H19Δ3 placenta. We found computationally predicted targets of miR-675 enriched in the genes upregulated in the H19Δ3 placenta (P = 0.0254). We co-transfected miR-675-5p and miR-675-3p mimics into ES, C2C12 and MEF cells and monitored the effect on 35 predicted targets by quantitative PCR with reverse transcription (qRT–PCR; Fig. 6a–c), many of which (45%) were found to be downregulated by miR-675. However, genes that seemed to be targets in one cell line were not necessarily targets in another. This suggests that the target network by which miR-675 slows cell proliferation is of a complex nature and may be distinct in different cell types.

**Igf1r is a potential target of miR-675**

Among the targets validated in cell lines, Igf1r was of particular interest because it is the key receptor through which Igf2 exerts its growth-promoting effect during fetal development. Igf1r is a potential target of miR-675-3p and contains two 7-mer seed matches in its 3′ UTR (Fig. 6d). We cloned the Igf1r 3′ UTR downstream of a luciferase reporter construct and co-transfected it into cells alongside either a miR-675-3p mimic or a scrambled control. We found that luciferase levels were reduced by more than 60% in the presence of miR-675-3p (Fig. 6e). This effect was not observed when the miR-675-3p-binding sites were mutated; thus, Igf1r is a potential target of miR-675-3p. Moreover, Igf1r was found to be upregulated in H19Δ3 placentas only at the time when miR-675 is expressed (E18.5) and not when it is silent (E11.5; Fig. 6f), suggesting that it is a target of the microRNA in vivo.

**DISCUSSION**

Our results suggest that a physiological role of H19 is to slow down growth of the placenta in the second half of gestation, in preparation for parturition. This seems to be achieved at least in part by downregulation of the RNA-binding protein HuR during gestation, which normally blocks processing of miR-675 at the Drosha stage. Increased levels of miR-675 in the placenta are concomitant with downregulation of Igf1r, amongst other targets that may also contribute to reduced growth. Igf1r has been shown to be an important regulator of growth31,32, with its main ligand during development being Igf2. Remarkably, the H19 locus therefore regulates the abundance of Igf2 (through imprinting) and that of its receptor, Igf1r, through miR-675.

Although data presented here apply to growth regulation in the placenta under physiological conditions, it is notable that in embryonic tissues miR-675 remains tightly repressed despite the
high levels of H19. We speculate that the enormous reservoir of the growth-suppressing miR-675 could be rapidly mobilized in response to cellular stress or abnormal proliferation. Indeed, H1R is known to relocate from the nucleus to the cytoplasm in stress conditions33–36 and on pharmacologically induced neoplastic transformation37. This would expose H19 to processing by Drosha, thus liberating miR-675. H1R was recently shown to suppress processing of human miR-7 from the highly expressed HNRNPK transcript38. That miR-7 is also a known tumour suppressor39–41 that functions partly through Igf1r targeting41 may suggest a wider miRNA repertoire for a H1R-mediated response to aberrant cell proliferation. The H19 RNA being a regulatable reservoir of the proliferation-suppressing miR-675 may thus explain the lincRNAs tumour-suppressive role, especially in childhood tumours42. The mechanisms of processing and function of miR-675 are likely to be relevant to the molecular pathology of fetal growth and cancer syndromes.

METHODS

Methods and any associated references are available in the online version of the paper at www.nature.com/naturecellbiology

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AUTHOR CONTRIBUTIONS

A.K. designed and carried out experiments and interpreted results. D.O. performed mass spectrometry. P.M. collected and dissected placentas for RNA sequencing. M.K. made the A2lox-cre ES cell line. L.D., G.S. and W.R. designed and supervised this study. All authors participated in writing the manuscript and reviewing its contents.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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METHODS

Cell culture. ES cells were the J1 line and were maintained on gelatinized dishes in ES media (Dulbecco’s modified Eagle’s medium (DMEM) containing sodium pyruvate and l-glutamine, supplemented with 15% fetal bovine serum (Invitrogen), 100 µg/ml penicillin/streptomycin, 1 x non-essential amino acids, 0.05 mM 2-mercaptoethanol and leukemia inhibitory factor (LIF, 1,000 units/ml; Millipore)).

TS cells were the TS-rs26 line derived in the Rossant Laboratory, cultured in standard conditions (RPMI 1640 supplemented with 20% fetal bovine serum (Invitrogen), 1% antibiotic/antimycotic solution, 1 x sodium pyruvate and 0.05 mM 2-mercaptoethanol, 2% fetal growth factor (bFGF, Sigma) and 1 µg/ml heparin (Sigma) was 70% of the media preconditioned with embryonic feeder cells48). Differentiation was achieved by culturing without bFGF or heparin in unconditioned media.

C2C12 cells were cultured in growth media (DMEM) supplemented with 200 mM l-glutamine and 10% fetal bovine serum (PA)). Differentiation was induced by culturing in differentiation media (DMEM supplemented with 200 mM l-glutamine and 2% horse serum (Invitrogen)).

MEFs were derived from C57/BL6 x CBA mice and cultured in ES cell media without LIF. H2R−/− MEFs have been previously described49. RNA was isolated from cells using the Qiazol method (Qiagen).

Animal studies. Mice used for the expression studies were the C57/BL6 strain. The H1947 transgenic line has been previously described47. All experiments were performed under Home Office licence and in accordance with the Animals (Scientific Procedures) Act of 1986. To define developmental stage, the day of conception is considered to be day 0.5 of pregnancy.

For absolute quantification of H19 levels, part of H19 was amplified using the H19 reverse transcription primers (see Supplementary Table S2) and cloned into the pGEM T-easy vector (Promega). Standard curves were created by qRT–PCR using a dilution series of this vector that was used to measure absolute H19 levels in tissue samples.

Northern blotting. To detect pre-miR-675, probes were PCR amplified from H19-containing plasmid DNA using a forward (5′-TGGCGGCCAGGAGGACTGTT-3′) and reverse primer harbouring the T7 promoter (5′-GGATCCTAATACGACTCACTATAGGGAGAGGAGCCAGACCCAGGACTGTA-3′). PCR products were reverse transcribed to generate radiolabelled probes. RNA was labelled using [α-32P]UTP. Total RNA was separated on a 15% TBE-Urea denaturing gel (Invitrogen). RNA was transferred to a GeneScreen Plus Hybridization Transfer membrane (Perkin Elmer) and incubated with labelled probe or labelled U6 RNA oligonucleotide (5′-TTGGGTGTACCTCTTGTAGGGAG-3′) for approximately 16 h. Membranes were exposed to X-ray film (Fujifilm).

For detection of mature miR-675, total RNA was separated on a 15% TBE-Urea denaturing gel (Invitrogen). RNA was transferred to a GeneScreen Plus Hybridization Transfer membrane (Perkin Elmer) and ultraviolet crosslinked. Probes specific to miR-675-3p (Exiqon) or U6 RNA were labelled with ATP[γ-32P] and incubated with membrane for approximately 16 h before exposure to X-ray film (Fujifilm).

mRNA library preparation (RNA sequencing). RNA sequencing libraries were created. sequenced and mapped using an in-house protocol that has already been described44. Mapped sequence reads were analysed with the SeqMonk Mapped Sequence Analysis Tool (http://www.bioinformatics.bbsrc.ac.uk/projects/seqmonk/). These data are available on the ArrayExpress database (http://www.ebi.ac.uk/arrayexpress/) with accession number E-MTAB-895. Sequencing data for miR-675 can be found on the mirBase database (http://www.mirbase.org/) with accession number MM004123.

mRNA affinity assay. Bait regions were PCR amplified from genomic DNA using appropriate primers (miR-675 stem: 5′-AATGGAAAAAGGCGATCTG-3′ and 5′-CCCGACCTGCTGCTACTCT-3′, 5′ H19: 5′-AGACCTGCGACGATGAA-3′ and 5′-GCCCAGCTGCTGCAAGGACT-3′, and 5′-GCCCTGCTGCAAGGACT-3′, and Kcnq1ot1:5′-TGGGGCGCTCCTAATACGTTG-3′ and 5′-CTGGGGCGCTCCTTGTACGG-3′) to produce amplicons of 343, 363 and 411 bp respectively. Note that the miR-675 stem fragment includes the putative H2R-binding site adjacent to the miR-675 stem loop. PCR products were cloned into the pGEM T-easy vector (Promega) and digested with Scal (New England Biolabs) to define the 3′ end before in vitro transcription.

RNA-binding proteins were then isolated using previously described methods42. Proteins were identified by mass spectrometry. Briefly, Coomassie-stained gel lanes were excised, destained, reduced, carbamidomethylated and digested overnight with 10 ng/ml modified trypsin (Promega) in 25 mM ammonium bicarbonate at 30 °C. The resulting peptide mixtures were separated by reverse-phase liquid chromatography (column: 0.05 x 100 mm, Vydac C18, 5 mm particle size), with an acetonitrile gradient (5–40% over 30 min) containing 0.1% formic acid, at a flow rate of 150 nl/min-1. The column was coupled to a nanospray ion source (Protaba Engineering) fitted to a quadrupole–time-of-flight mass spectrometer (Qstar Pulsar i; Applied Biosystems/MDS Sciex), operating in information-dependent acquisition mode.

RNA immunoprecipitation. Cells were cultured from either cultured MEF cells or mechanically homogenized whole placenta by incubation in lysis buffer (100 mM KCl, 5 mM MgCl2, 10 mM HEPES, at pH 7, 0.5% NP-40 and 1 x Complete mini Protease Inhibitor Cocktail tablets (Roche)). RNA immunoprecipitation was performed using 4 µg of antibody against H2R (3A2, Santa Cruz) or non-specific mouse IgG (Santa Cruz) and a previously described method46.

Luciferase assay. The miR-675 target sites within the Igf1r were PCR amplified using primers containing artificial XhoI and NotI restriction sites and cloned into the Psychock2 vector (Promega) directly downstream of a luciferase reporter. Other miRNA-binding sites were mutated by PCR amplifying the Psychock2–Igf1r vector with primers containing the required point mutations followed by removal of un-mutated plasmid by DpnI digestion. Plasmids (200 µg) were co-transfected into ES cells with miR-675 mimic (24 nM, Ambion) and the luciferase activity measured 24 h later under the Dual Glo luciferase assay (Promega).

TUNEL staining. Cells were cultured with Pre-miR miRNA precursor molecules (Ambion), as described above, and maintained for 8 h before being collected and cytospun onto poly-L-lysine-coated slides. Apoptotic cells were detected using the DeadEnd Fluorometric TUNEL system (Promega). Positive controls
were made by treatment with DNaseI (Roche) according to instructions supplied with the TUNEL staining kit. Cell nuclei were stained with Vectashield with DAPI (Vector Laboratories) and visualized on an Olympus BX41 fluorescence microscope.

### Nuclear cytoplasmic RNA fractionation.

Nuclear and cytoplasmic RNA was extracted from MEF cells with a PARIS kit (Ambion). Fractionated placental RNA was obtained by mechanical homogenization of placentas in lysis buffer (0.32M sucrose, 5 mM CaCl$_2$, 5 mM EDTA, 3 mM MgAc, 10 mM Tris–HCl at pH 8 and 40 U ml$^{-1}$ RNasin (Promega)) and filtration. Placental lysate was then centrifuged at 700g for 10 min and the supernatant retained as the cytoplasmic fraction for RNA extraction. Nuclei were resuspended in centrifuge buffer (2M sucrose, 3 mM MgAc and 10 mM Tris–HCl at pH 8) and overlaid on a cushion of further centrifuge buffer before centrifugation at 26,000g for 1 h and RNA extraction.

### Statistical analysis.

Data were analysed either by two-way analysis of variance followed by a post hoc Tukey test, or a two-tailed Student $t$-test. $P < 0.05$ was considered as significant.

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**Figure S1** Imprinted status of miR-675 and expression in cell lines. (a) The expression of miR-675 in E19.5 placenta of mice lacking maternal H19 (H19^{Δ13+/-mat}) determined by qRT-PCR reveals that miR-675 is not expressed from the paternal allele. n = 2. (b,c) TSCs were differentiated for 2 days into cultures rich in spongiotrophoblast-like cells (ST), and for a further 2 days into giant cell rich cultures (Giant). (b) qRT-PCR results for marker genes of TS cell multipotency (Cdx2) and differentiation (Csh1 and Csh2). (c) Expression of both species of miR-675 and H19, displayed as a percentage of expression levels observed in the E19.5 placenta. n = 2. (d) Expression of both species of miR-675 and H19 in MEF cells, expressed as a percentage of their RNA levels observed in the E19.5 placenta. n = 3. Error bars show the s.e.m.
Figure S2 siRNA mediated knockdown of candidate miR-675 inhibitory factors in MEF and C2C12 cells. Cells were treated with either an siRNA designed to knockdown the mRNA of each individual candidate protein, or a scrambled control and RNA measurements made by qRT-PCR. (a, c) The efficiency of knockdown achieved on each candidate mRNA is shown for MEF cells (a) and C2C12 cells (c). (b, d) The effect of candidate knockdown on both species of miR-675 and miR-16 is shown for MEF cells (b) and C2C12 cells (d). Note that the si-HuR data is the same as appears in the main text in Fig. 2e, g. n = 4. Error bars show the s.e.m. Statistical significance was determined by Student’s t-test. * P < 0.05, ** P < 0.01, *** P < 0.001.
Figure S3 miR-675 does not induce apoptosis. (a) G401 cells were transfected with either miR-675-5p, a scrambled control or mock transfected with lipofectamine. Cell nuclei were stained with DAPI (blue) and apoptotic cells marked by TUNEL staining (green). The merged overlay of DAPI and TUNEL stained images is also shown. As a positive control, cells were treated with DNaseI to damage DNA and allow TUNEL staining. Scale bars indicate 50 µm. Note that similar results have been obtained for ES, TS, MEF and C2C12 cells but the data is not shown. (b) Expression of Rb1 mRNA, detected by qRT-PCR, following the co-transfection of both miR-675 species mimics or a scrambled negative control in ES, C2C12 and MEF cells. n = 3. Error bars show the s.e.m. Statistical significance was determined by Student's t-test. * P < 0.05, ** P < 0.01.
Figure S4  Doxycycline induction of miR-675 in A2lox.cre ES cells. Using the A2lox system, ES cells were modified to stably express either miR-675-5p (A2lox-5p), miR-675-3p (A2lox-3p) or a scrambled control (A2lox-scrambled) from a doxycycline inducible promoter. (a, b) miR-675-3p (a) and miR-675-5p (b) expression in the modified cell lines, both in the presence and absence of doxycycline, determined by qRT-PCR. (c) Expression of both species of miR-675 in the modified ES cell lines relative to the expression levels observed in the E19.5 placenta. Data shown is from 2 individual cell lines and 4 independent experiments for each modification. Error bars show the s.e.m.
**Figure S5** Expression of H19 and miR-675 in differentiating C2C12 cells. The myoblast cell line C2C12 was differentiated for 96 hours (h) towards myotubes and RNA measured by qRT-PCR. (a) Expression of marker genes for TS cell multipotency (Pax7), early differentiation (Myog) and myotubes (MCK). (b) Expression of both species of miR-675 and H19, expressed as a percentage of expression levels observed in the E19.5 placenta. n = 2.
Figure S6  Gene expression and phenotype of the $H19^{\Delta3}$ mouse. (a) qRT-PCR validating the $Igf2$ expression levels observed in the E18.5 $H19^{\Delta3}$ placenta. $n = 5$. Error bars show the s.e.m. (b) RNA-seq data showing $H19$ expression relative to $Gapdh$ and $Actb$ in the wild type E18.5 placenta. Error bars show the standard deviation between measurements of each exon. (c) Embryonic weight of $H19^{\Delta3}$ mice from homozygous crosses compared to embryonic weights of wild type embryos. The number of embryos measured for each data point is indicated above each bar. Error bars indicate the s.e.m. p-values were determined by Student’s t-test. ** $p < 0.01$, *** $p < 0.001$. 
**Figure S7** Expression of H19 and miR-675 in dissected placentas and the H19\textsuperscript{Δ3} placental transcriptome. (a) Diagrammatic representation of the murine placenta. The labyrinthine zone (LZ) and junctional zone (JZ) are marked. (b) qRT-PCR results for marker genes of the JZ (Tpbpa) and LZ (Syna) from E18.5 placental samples dissected into layers. (c) Expression of both species of miR-675 and H19 in dissected E18.5 placentas, displayed in relative arbitrary units. (d) Expression of both species of miR-675 and H19 in dissected placental samples, displayed in molecules per ng of total RNA, such that comparison between the three RNA species can be made. n = 5. Error bars indicate the s.e.m. Statistical significance was determined by Student’s t-test. ** P < 0.01, *** P < 0.001. (e) RNA-seq data from the labyrinth layer of E18.5 wild type and H19\textsuperscript{Δ3} placenta displayed as a scatter plot. Genes differentially regulated more than 2-fold are indicated.
Figure S8 Full scans of northern blots shown in Fig 3a, b.