**Prenatal correction of IGF2 to rescue the growth phenotypes in mouse models of Beckwith-Wiedemann and Silver-Russell syndromes**

**Highlights**
- Prenatal intervention is successful in BWS/SRS mouse models by targeting IGF2
- A pharmacological rescue is available to prenatally normalize IGF2 signaling in BWS
- Placenta growth indicates subsequent fetus growth anomalies in BWS/SRS models
- IGF2 peptide in the amniotic fluid is a diagnostic biomarker for BWS and SRS models

**In Brief**
Liao et al. use mouse models to test a prenatal approach for correcting growth anomalies in two imprinting diseases, BWS and SRS. They find that cases where the fetal growth factor IGF2 is misregulated can be diagnosed, and growth can be corrected by prenatally adjusting IGF2 or its signaling output.

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Liao et al., 2021, Cell Reports 34, 108729
February 9, 2021 © 2021 The Author(s).
https://doi.org/10.1016/j.celrep.2021.108729
Prenatal correction of IGF2 to rescue the growth phenotypes in mouse models of Beckwith-Wiedemann and Silver-Russell syndromes

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SUMMARY

Beckwith-Wiedemann syndrome (BWS) and Silver-Russell syndrome (SRS) are imprinting disorders manifesting as aberrant fetal growth and severe postnatal-growth-related complications. Based on the insulator model, one-third of BWS cases and two-thirds of SRS cases are consistent with misexpression of insulin-like growth factor 2 (IGF2), an important facilitator of fetal growth. We propose that the IGF2-dependent BWS and SRS cases can be identified by prenatal diagnosis and can be prevented by prenatal intervention targeting IGF2. We test this hypothesis using our mouse models of IGF2-dependent BWS and SRS. We find that genetically normalizing IGF2 levels in a double rescue experiment corrects the fetal overgrowth phenotype in the BWS model and the growth retardation in the SRS model. In addition, we pharmacologically rescue the BWS growth phenotype by reducing IGF2 signaling during late gestation. This animal study encourages clinical investigations to target IGF2 for prenatal diagnosis and prenatal prevention in human BWS and SRS.

INTRODUCTION

Beckwith-Wiedemann syndrome (BWS; OMIM 130650) and Silver-Russell syndrome (SRS; OMIM 180860) are imprinting disorders (Monk et al., 2019) that display fetal overgrowth and fetal growth retardation, respectively. Most BWS symptoms (Brioude et al., 2018) directly or indirectly depend on aberrant growth regulation during gestation: large birth weight; enlarged placenta, tongue, kidney, and liver; and childhood cancers associated with overgrowth (Brioude et al., 2019), such as Wilms tumor of the kidney. A total of 68% of BWS children undergo at least one surgery related to BWS, including tongue or kidney reduction or cancer treatment (McNeil et al., 2002; Naujokat et al., 2019; Style et al., 2018). SRS symptoms (Wakeling et al., 2017) that depend on aberrant growth regulation include small for gestational age (SGA), relative macrocephaly at birth, severe feeding difficulties, and low body mass index.

The opposite growth anomalies of BWS and SRS correspond to the reciprocal genetic and epigenetic (DNA methylation) defects of the same genetic region (Eggermann, 2009). Approximately 80% of BWS and 60% of SRS cases have a molecular defect at chromosome 11p15.5 (Kalish et al., 2012) that hosts two imprinted domains. In domain 1, the paternally expressed insulin-like growth factor 2 (IGF2) gene, regulated by the imprinting control region 1 (ICR1), promotes growth, and in domain 2, the maternally expressed cyclin-dependent kinase inhibitor 1c (CDKN1C), regulated by the imprinting control region 2 (ICR2), inhibits growth. The phenotypes of BWS and SRS can be effectively modeled in the mouse (Chang and Bartolomei, 2020). Cdkn1c mutant mice do not exhibit neonatal overgrowth (Tunster et al., 2011), whereas models with Igf2 misregulation manifest in fetal and neonatal BWS-like overgrowth or SRS-like growth retardation (Singh et al., 2012).

IGF2 encodes the fetal growth factor IGF2. Its RNA is expressed in the chromosome inherited from the father in mice and humans (DeChiara et al., 1991; Giannoukakis et al., 1993). Our knowledge regarding mechanisms that regulate the paternal allele-specific expression of IGF2 is based on genetic studies that focused on the orthologous mouse Igf2-H19 imprinted domain, as summarized in Figure 1A (Singh et al., 2012). IGF2 regulates the supply of nutrients by the placenta and also the demand for nutrients by the fetus (Constancia et al., 2002). H19 is a non-coding RNA expressed from the maternally inherited chromosome (Bartolomei et al., 1991; Ferguson-Smith et al., 1993). The H19 RNA releases miR-675 at late gestation to suppress the growth of the placenta just before birth (Keniry et al., 2012). The genomic imprint of this domain is inherited from the father and consists of CpG methylation along the ICR1 (Thorvaldsen et al., 2001a).
In the soma, ICR1 functions as a DNA-methylation-sensitive insulator (Figure 1A). We and others reported that four CTCF molecules bind in the unmethylated maternal ICR1 allele to insulate the \textit{Igf2} gene from its enhancers, but in the paternal allele, CTCF binding is prevented by DNA methylation, allowing \textit{Igf2} transcription (Bell and Felsenfeld, 2000; Hark et al., 2000; Kaffer et al., 2000; Kanduri et al., 2000; Szabó et al., 2000).

BWS molecular defects at the ICR1 can be genetic or epigenetic (Brioude et al., 2018). The insulator model (Figure 1A) predicts that in each of these BWS classes (Figures S1A–S1C; Azzi et al., 2014; Bachmann et al., 2017; Brioude et al., 2018; Netchine et al., 2013; Riccio et al., 2009) \textit{IGF2} is transcribed from two chromosomes instead of one, resulting in an elevated dose of \textit{IGF2} protein, which causes the overgrowth symptoms. In mouse genetic models, an increased dose of \textit{Igf2} recapitulated the overgrowth phenotypes of BWS. These models include the \textit{Igf2} transgenic mice (Eggenschwiler et al., 1997; Sun et al., 1997), mice with paternal duplication of distal chromosome 7 (PatDup.dist7) (Rentsendorj et al., 2010), and mice that carry mutation in the CTCF binding sites in the maternal ICR1 allele (Szabó et al., 2004). Elevated and biallelic \textit{IGF2} RNA expression was found in the tongue and fibroblast of human BWS patients (Weksberg et al., 1993; Reik et al., 1995), supporting the insulator model in humans.

The complex genetic and epigenetic molecular defects found in SRS at the ICR1 (Figures S1D and S1E; Azzi et al., 2014; Begemann et al., 2015; Eggermann et al., 2006; Gicquel et al., 2005; Peñaherrera et al., 2010; Wakeling et al., 2017; Liu et al., 2017; Yamoto et al., 2017) are consistent with reduced \textit{IGF2} expression and fetal growth retardation. Mice that carry an \textit{Igf2} null mutation are 60% the size of normal siblings (DeChiara et al., 1990). Very small size also characterizes the embryos carrying maternal duplication of distal chromosome 7 MatDup.dist7 (Han et al., 2010) and fetuses that are engineered to exhibit biallelic ICR1 insulation (Lee et al., 2010; Szabó et al., 2002; Engel et al., 2004), due to greatly reduced \textit{Igf2} levels. Reduced \textit{IGF2} RNA levels in human SRS fibroblasts (Gicquel et al., 2005) and epimutation positive fetuses and placentas (Yamazawa et al., 2017) were also observed in SRS.

See also Figure S1.
The SRS model (+/CHB) (Lee et al., 2010) was generated by recombination of the unmethylated chicken enhancer insulation (Figure 1B), resulting in fetal overgrowth. The goal of the current work was to address three clinical challenges: (1) IGF2, a known facilitator of fetal growth, has not been targeted in clinical therapy of BWS or SRS; (2) BWS and SRS children are normally diagnosed after birth, when most of the symptoms have already developed and IGF2 levels can no longer be corrected efficiently; and (3) the management of these diseases does not include prenatal intervention. To address these challenges, we used mouse models to derive proof-of-principle evidence showing that IGF2-based diagnosis and therapy at the fetal stage could prevent the overgrowth symptoms of BWS and the growth retardation of SRS.

RESULTS

Animal models of BWS and SRS

We previously generated gene-targeted mouse lines to model the molecular features of BWS and SRS cases that are related to the ICR1 (Lee et al., 2010; Szabó et al., 2002, 2004). The BWS model (CTCFm/+), the mother’s genotype is always written first) was created by introducing point mutations in the four CTCF binding sites (CTCFm allele) in the maternal ICR1 allele (Szabó et al., 2004). IGF2 is biallelically expressed in the absence of enhancer insulation (Figure 1B), resulting in fetal overgrowth. The SRS model (+/CHB) (Lee et al., 2010) was generated by replacing the methylated, paternal H19 ICR1 with tandem copies of the unmethylated chicken β-globin insulator region (CHB), which contains two functional CTCF binding sites and insulates IGF2 in the paternal chromosome (Lee et al., 2010; Szabó et al., 2002; Figure 1C). Biallelic ICR1 insulation results in undetectable IGF2 and in turn causes fetal growth retardation.

A double genetic rescue corrects growth anomalies of the BWS and SRS models

We predicted that a paternally inherited CHB allele rescues the growth retardation of a paternally inherited CTCFm allele. At the same time, a maternally inherited CTCFm allele should rescue the growth retardation of a maternally inherited CHB allele. We generated a rescue mouse model, CTCFm/CHB (Figure 1D), which is a compound heterozygote of the mutant alleles from the BWS model (CTCFm/+ ) and SRS model (+/CHB). We crossed +/CTCFm mothers with CHB/+ fathers to obtain offspring of four genotypes (Figures 1A–1D). We examined the resulting weanlings at 21 days postpartum (dpp), the newborns at 1 dpp, and fetuses at 18.5 days postcoitum (dpc) (Figures 1E–1G). We found three phenotypes and four genotypes: large (+/+), small (+/CHB), and normal (+/+ or CTCFm/CHB) (Figure 1C). Biallelic ICR1 insulation results in undetectable IGF2 and in turn causes fetal growth retardation.

The weights of CTCFm/+ and +/CHB weanlings were significantly larger in the CTCFm/+ than in the +/+ pups, whereas these organs were significantly smaller in the +/CHB than in the +/+ pups (Figures 3A and 3B). The heart, kidney, liver, lung, and tongue organs were significantly larger in the CTCFm/+ than in the +/+ pups, whereas these organs were significantly smaller in the +/CHB than in the +/+ pups (Figures 3A and 3B). However, the weight of each organ of the CTCFm/CHB pups was indistinguishable.
from the +/+ pups (Figures 3A and 3B), except for the tongue that was slightly larger in the CTCFm/CHB pups.

We found that most of the internal organs were proportional to the body weight in both models, that is the relative organ/body weights of CTCFm/+ and +/CHB pups did not differ significantly from the +/+ pups, except for the brain and the tongue (Figure 3C; Figure S3). The brain/body ratio was slightly smaller in the CTCFm/+ pups and it was larger in the +/CHB pups than in the +/+ pups. These results collectively demonstrate that the generalized organ weight anomalies found in the heart, kidney, liver, and lung of the BWS and SRS models can be corrected genetically by normalizing ICR1 insulation. In addition, the relative microcephaly and relative macrocephaly growth phenotypes of the IGF2-dependent BWS and SRS models, as well as the relative macroglossia of the BWS model, can also be corrected.

The double genetic rescue normalizes IGF2 levels at the fetal stage

We examined the underlying molecular mechanism of growth normalization in the compound heterozygous mice. Because both the CTCFm and CHB mutations misregulate the imprinted Igf2 and H19 genes, we expected that the misexpressions of these genes are the major cause of the body and organ size aberrations in the mutant genotypes. We collected 11 organs at 18.5 dpc and measured Igf2 and H19 transcript levels by using quantitative reverse-transcription PCR (Figures 4A and S4A) and quantified parental allele-specific transcription using multiplex allelotyping assays (Figures 4B and S4B). We found that the organ sizes at 21 dpp generally corresponded to the Igf2 transcript levels at 18.5 dpc. The heart, kidney, liver, lung, and tongue organs were large, small, and normal-sized, respectively, in the CTCFm/+ and +/CHB, and CTCFm/CHB weanlings (Figure 3A), and these organs exhibited 2-fold higher, greatly reduced, and normal Igf2 RNA expression levels, respectively, at 18.5 dpc (Figure 4A). The liver was an exception, as its size was normalized in the rescue model at 21 dpp, but larger in CTCFm/+ than in +/+ pups. However, it was not significantly different between the CTCFm/CHB and +/+ pups.

Figure 2. Genetic rescue corrects the growth phenotypes of BWS and SRS models

(A) Boxplot shows the weight distribution of pups on the day of weaning, +/+ (n = 12), CTCFm/+ (n = 14), +/CHB (n = 7), and CTCFm/CHB (n = 16). The data were collected from eight litters of +/CTCFm females crossed with CHB/+ males. (B) Postnatal weight gain of individual pups from two litters is plotted by genotype from birth to weaning at 21 dpp. (C and D) Boxplots depict the distribution of fetus (C) and placenta (D) weights for early (13.5 dpc) and late (18.5 dpc) fetal stages. The numbers of fetuses and placentas collected from multiple litters were as follows: +/+ (n = 11), CTCFm/+ (n = 11), +/CHB (n = 17), and CTCFm/CHB (n = 16) at 13.5 dpc; and +/+ (n = 10), CTCFm/+ (n = 16), +/CHB (n = 6), and CTCFm/CHB (n = 6) at 18.5 dpc. The significant differences between mutant and +/+ samples are indicated. See also Figure S2.
Igf2 transcript levels were not normalized at 18.5 dpc (Figure 4A). Postnatal liver weight is, therefore, partially independent of fetal Igf2 levels. The +/+ organs expressed Igf2 from the paternal allele except the brain, which expressed biallelic Igf2 (Figure 4B), as expected (DeChiara et al., 1991). In agreement with the insulator model, elevated Igf2 transcript levels in the CTCFm/+ fetus organs (Figure 4A) corresponded to biallelic Igf2 transcription (Figure 4B). The brain of +/CHB pups was slightly but significantly smaller than normal (Figure 3A) and corresponded to reduced Igf2 expression in the fetal brain (Figure 4A). The brain sizes were normal in the CTCFm/+ and CTCFm/CHB genotypes (Figure 3A), and Igf2 expression levels were normal (Figure 4A). Relative microcephaly in the BWS model (Figure 3C) was consistent with the loss of imprinting of Igf2 in the body only but not in the brain, where biallelic Igf2 expression was unchanged (Figure 4B).

H19 and Igf2 genes belong to an imprinted gene network (IGN), and H19 long non-coding RNA (lncRNA) is a regulator of a large node of this IGN (Gabory et al., 2009; Monnier et al., 2013). To test whether the ICR1 affects allele-specific transcription beyond the H19/Igf2/Igf2as domain, we examined additional imprinted genes along distal chromosome 7 (Figure S5) and elsewhere (Figure S6). We found that, except for H19, Igf2, and Igf2as, the allele-specific expression of imprinted genes was undisturbed in the insulator mutants and in the compound heterozygotes. Igf2as, which is paternally expressed in the mouse (Moore et al., 1997), exhibited paternal expression in the +/+ organs except in the brain, where it was biallelic, and also showed biallelic expression in CTCFm/+ organs, similar to Igf2 (Figure S5). These results (Figures S5 and S6) collectively suggest that the effects of the CTCFm or CHB mutations on allele-specific transcription are confined to the H19/Igf2/Igf2as imprinted.
domain along chromosome 7. This is reassuring for future gene therapy approaches that may target IGF2 or the ICR1 for in utero treatment of BWS and SRS cases.

We found, using western blotting, that the secreted IGF2 (IGF2s) peptide level was elevated 4-fold in the amniotic fluid of the BWS model and was close to undetectable in the SRS model at 13.5 dpc (Figure 4C). Importantly, the level of the IGF2 peptide was corrected to ~1-fold in the amniotic fluid of the compound heterozygous rescued fetuses. The level of the IGF2 precursor was also normalized in the amnion and the kidney. In summary, the BWS and SRS growth phenotypes were rescued in the CTCFm/CHB mice, which displayed corrected Igf2 RNA and IGF2 protein levels (Figures 4A and 4C).

The IGF2-dependent BWS and SRS models are molecularly very similar to human BWS and SRS anomalies that predict IGF2 misexpressions (Figure S1); therefore, such human cases can be predicted to respond to the normalization of IGF2 levels or IGF2 signaling. Even though IGF2 has long been considered the culprit in both BWS and SRS, IGF2 is not targeted in therapy. In human children, BWS and SRS are normally diagnosed and managed only after birth. Next, we present evidence in the mouse that the fetal stage is optimally suited for diagnosis and intervention in IGF2-dependent BWS and SRS because IGF2 activity is specific to the fetal stage.

We measured Igf2 transcript levels in the organs of the 18.5-dpc fetuses using quantitative reverse-transcription PCR (Figure 5A). We found that Igf2 levels were much higher in the extraembryonic organs (placenta, yolk sac, and the amnion), than in the organs of the fetus body (liver, kidney, tongue, brain, heart, lung, muscle, and stomach). Among fetal organs, the liver exhibited the highest expression of Igf2, which plummeted after birth following a brief peak at 7 dpp and remained low during adulthood (Figure 5B). Igf2 did not exhibit the brief postnatal surge in the kidney and brain. Postnatal downregulation of Igf2 mRNA was also reported in the heart, muscle tongue, and lung (Weber et al., 2001; Constancia et al., 2000; Lui et al., 2008). This expression pattern suggests that the IGF2 protein exerts its major growth-promoting effect during fetal development while the extraembryonic organs persist. It also implies that any intervention or diagnosis that targets IGF2 will be successful prenatally but not much longer after birth.

We found that the difference in fetus weights between CTCFm/+ samples and +/+ samples greatly increased between 16.5 dpc and 18.5 dpc (Figure 5C). The placenta weights were already 50% higher in CTCFm/+ samples than in the +/+ samples at 16.5 dpc (Figure 5D). These results show that the aberrant placenta growth in the IGF2-dependent BWS model at 16.5 dpc is indicative of the subsequent fetus overgrowth that occurs except for the amniotic fluid. In the absence of cellular protein content, relative values were calculated based on Igf2 concentration per equal volumes of amniotic fluid in each sample. For additional imprinted genes please see Figures S4, S5, and S6.

The fetal stage presents a window of opportunity for diagnosing IGF2-dependent BWS and SRS growth aberrations

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between 16.5 and 18.5 dpc. Thus, the late fetal stage represents a window of time when the IGF2-dependent BWS overgrowth may be intercepted.

**IGF2 in the amniotic fluid is diagnostic of the SRS and BWS models**

We performed western blotting using protein lysates from organs of a 13.5-dpc fetus and found that the full-length IGF2 protein, at ~18 kilodaltons (kDa), was clearly detectable in the amnion, yolk sac, and kidney; whereas the secreted IGF2 peptide (~8 kDa) was abundant only in the amniotic fluid (Figure 5E). Next, we explored the possibility of using IGF2 peptide level in the amniotic fluid as a fetal diagnostic marker in IGF2-dependent BWS and SRS. We found that the amniotic fluid had extremely low IGF2 peptide concentration in the SRS model (+/CHB) at 13.5 dpc (Figure 4C), at the time when the placenta weights indicated the SRS phenotype (Figure 5D). IGF2 peptide level was 4 times higher in the CTCFm/+ than in the +/+ amniotic fluid at 16.5 dpc (Figure 5F), when the placenta weights indicated the BWS phenotype (Figure 5D). These results show that IGF2 peptide levels in the amniotic fluid can be used as diagnostic targets in the SRS and BWS models at 13.5 and 16.5 dpc, respectively.

**Pharmacological rescue of the BWS overgrowth phenotype**

The results of the genetic rescue experiment were consistent with the idea that correcting IGF2 levels normalizes the growth anomalies in the BWS model. We next explored the possibility of a pharmacological intervention in BWS. IGF2 signaling is mediated by the IGF1 receptor (IGF1R) (Baker et al., 1993; Bergman et al., 2013; Gicquel and Le Bouc, 2006). Picropodophyllin (PPP) is a drug approved by the Food and Drug Administration (FDA) to block IGF2 signaling. It is known to inhibit IGF1R kinase activity (Girnita et al., 2004). We treated CTCFm/+ and +/+ fetuses in utero with 20 mg/kg PPP for 2 days (starting on 16.5 dpc) or 5 days (starting on 13.5 dpc) and measured the weight...
Figure 6. Prenatal pharmacological rescue of the BWS model

(A and B) Prenatal treatment by the IGF1R inhibitor picropodophyllin (PPP) antagonizes the IGF2 overdose and prevents fetal overgrowth in the BWS model fetuses. Boxplots show fetus (A) and placenta (B) weights from PPP-treated and control mothers. PPP was administered orally to CTCFm/+ pregnant dams at 20-mg/kg dose for 2 days, starting at 16.5 dpc CTCFm/+ (n = 15) and ++ (n = 14), 4 litters, or for 5 days, starting at 13.5 dpc CTCFm/+ (n = 19) and ++ (n = 16), 8

Cell Reports 34, 108729, February 9, 2021

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of the fetuses and placentas at 18.5 dpc. We found a statistically significant reduction in the weight of PPP-treated CTCFm/+ fetuses (Figure 6A) compared with untreated CTCFm/+ controls after 2 days or 5 days of PPP treatment. The weight of CTCFm/+ fetuses was indistinguishable from that of +/+ control fetuses after 5 days of PPP treatment, suggesting that fetus weight was corrected in those mutants by PPP. The weight of +/+ fetuses was also significantly reduced after 2 days or 5 days of PPP but to a lesser extent than the CTCFm/+ fetuses. The weight of CTCFm/+ and +/+ placentas (Figure 6B) did not change significantly after 2 or 5 days of PPP, suggesting that contrary to what we found in the fetus, blocking the IGF1R does not reduce IGF2 activity in the placenta.

We also addressed the postnatal growth effects of PPP. The body weight of PPP-treated CTCFm/+ pups was significantly smaller than that of the untreated CTCFm/+ pups at the weaning age (Figure 6C) and was indistinguishable from the untreated +/+ pups, consistent with a rescued growth phenotype. Surprisingly, the weight of PPP-treated +/+ pups was not significantly smaller than the untreated +/+ pups (Figure 6C), suggesting that they can catch up in growth after the initial reduction at the fetal stage (Figure 6A). We then examined the organ weight at 21 dpp (Figure 6D). The PPP-treated CTCFm/+ heart was indistinguishable from the heart of the control +/+ pups, showing a complete rescue. The kidney and lung organs of the PPP-treated CTCFm/+ pups were significantly smaller than those of the CTCFm/+ control pups, although they were still slightly larger than those of the control +/+ pups, consistent with a partially rescued growth phenotype in those organs. The tongues of the PPP-treated and control CTCFm/+ pups were not significantly different. However, the difference between CTCFm/+ and +/+ tongues were highly significant (p = 0.0002), but the difference between PPP-treated CTCFm/+ and control +/+ tongues were hardly significant (p = 0.048), consistent with a partially rescued growth phenotype in the tongue as well (Figure 6D). PPP treatment did not result in reduced liver weight at weaning, and this can be related to the brief postnatal peak of Igf2 transcription observed specifically in the liver (Figure 5B) or the independence of liver from IGF1R signaling. Because PPP affected the body weight but not the liver weight, the relative liver/body weights of CTCFm/+ and +/+ pups have increased after PPP (Figure 6E). The head/body (relative microcephaly) and tongue/body (relative macroglossia) phenotypes were corrected by the PPP intervention (Figure 6E). Importantly, none of the organs of PPP-treated +/+ pups changed significantly compared with the untreated controls. These results collectively suggest that most of the BWS-like overgrowth phenotypes can be intercepted by prenatal pharmacological intervention.

To confirm the mechanism of how PPP treatment rescues BWS overgrowth, we tested the effects of PPP on IGF1R signaling in the treated fetuses (Figure 6F). IGF2 binds to IGF1R and induces its autophosphorylation, which activates the Ras-mitogen-activated protein kinase (MAPK) and PI 3-kinase signaling pathways (Hakuno and Takahashi, 2018). In semiquantitative western blots, we found an increased level of phospho-IGF1R in the CTCFm/+ compared with that of the +/+ fetal kidneys at 18.5 dpc. The level of phospho-IGF1R was reduced in the PPP-treated CTCFm/+ samples compared with that of the control CTCFm/+ samples. In addition, we also found a reduction in phospho-MAPK (ERK1/2) and phospho-AKT in the PPP-treated samples compared with the control samples. These findings support the proposal that CTCFm/+ overgrowth in the kidney is mediated by the IGF2-IGF1R axis and that PPP corrects the overgrowth by interfering with IGF2 signaling.

We did not find any adverse effects of PPP treatment on the mothers. The animals were monitored daily, and no behavioral or physical abnormalities were observed during and after the treatment. All of the PPP-treated mothers named and the pups until the weaning day. The weight gain of PPP-treated CTCFm/+ pregnant females was similar to that of the control mothers (Figure S7A), and they became pregnant again within the next 2 months. The number of fetuses was similar for the first (PPP-treated) and second (untreated) pregnancies of these females (Figure S7B). In summary, we demonstrated that the IGF2-dependent BWS fetal overgrowth phenotypes can be prevented by prenatal pharmacological intervention.

**DISCUSSION**

In this work, we presented evidence by using our IGF2-dependent BWS and SRS mouse models in support of IGF2-based *in utero* diagnosis and intervention strategies. We showed that (1) the prenatal intervention through genetically correcting IGF2 is successful in these models at the late stage of gestation, (2) a proof-of-principle pharmacological method is available for normalizing IGF2 activity and fetal growth in the BWS model, (3) the placenta size anomalies are indicative of subsequent fetus growth anomalies in BWS and SRS, and (4) the secreted IGF2 peptide level in the amniotic fluid is a diagnostic biomarker for prenatal intervention. These results should encourage human studies in which BWS and SRS fetuses that exhibit IGF2 misexpressions are identified directly by measuring IGF2 peptide levels.

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4 litters. Control fetuses CTCFm/+ (n = 12) and +/+ (n = 12) from 3 litters were untreated. Adjusted p values are shown to indicate the significant differences of treated versus control samples of the same genotype.

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(C–E) Prenatal PPP prevents the overgrowth phenotypes of the weanling pups in the BWS model. Boxplots show the weight distribution of the total body (C), selected organ weights (D), and relative organ/body weights (E) on the day of weaning at 21 dpp. The significant differences between treated CTCFm/+ and control +/+ samples and between treated CTCFm/CHB and control +/+ are marked above horizontal bars. The significance values between treated and control samples of the same genotype are indicated above the boxes.

(F) PPP acts by blocking IGF1R signaling. Western blot of 18.5-dpc kidney samples. The protein lysates of two kidney samples, one female and one male, from each genotype and from each treatment group are displayed. Pan and phosphorylated IGF1R, AKT, and p44/42 MAPK(ERK1/2) antibodies were used. Fetus weights are provided at the bottom in the top panel. GAPDH was the loading control. The signals were quantified from the images, and the averages of the duplicates are displayed as ratios of the altered conditions compared with the +/+ untreated samples.

See also Figure S7.
levels in the amniotic fluid and the IGF2 signaling output is adjusted in utero to normalize growth before birth.

What is realistic in prenatal management, in particular with respect to the window size between prenatal detection of SRS/ BWS and successful prenatal treatment? The fetal stage is the optimal time for IGF2-based intervention because of the timing and the location of IGF2 expression. We found that the extraembryonic organs have the highest IGF2 RNA and IGF2 protein levels, and these organs are only available at the fetal stage. We suggest that apart from the role of IGF2 in the placenta, the secreted IGF2 peptide supplied into the fluids surrounding the fetus by the amnion and yolk sac also has an important growth-promoting effect on the fetus. The majority of fetus growth takes place in the third trimester. Therefore, the last 3 months should be the most effective time window for intervention, considering that 5 days of PPP treatment was successful for correcting BWS overgrowth in the mouse.

Importantly, we suggest that the cases that would respond to IGF2 therapy could be identified by directly measuring IGF2 peptide levels. We found diagnostic 4-fold of normal IGF2 peptide levels in the amniotic fluid of the BWS model at 16.5 pc and undetectable IGF2 in the SRS model at 13.5 dpc, before the fetuses showed abnormal growth (Figures 4 and 5). Based on these results in the mouse, we propose that human prenatal diagnosis could be done successfully by measuring IGF2 in the amniotic fluid. IGF2 was detected in human amniotic fluid and exocoelomic fluid by different methods (Blahovec et al., 2001; Chard et al., 1994; Merimee et al., 1984; Nonoshita et al., 1994). The exact IGF2 levels are contradictory in these studies due to the differences in the methodologies used for measuring IGF2 (Blahovec et al., 2001; Chard et al., 1994; Miell et al., 1997; Nonoshita et al., 1994), collecting amniotic fluid (Jauniaux and Gulbis, 2000), and statistics (Verhaeghe et al., 1999). These measurements will need to be revisited using uniform assays and should include verification by western blot.

Who should be screened for IGF2 level in utero? We suggest that every familial BWS and SRS pregnancy case should be screened for IGF2 peptide level. Other cases should be screened if indicated by anatomical and/or molecular defects characteristic of BWS and SRS that include ICR1 (Figure S1). Quantifying IGF2 levels in the amniotic fluid will reveal whether IGF2 levels are abnormal and also how much adjustment is necessary. We showed in the mouse that placenta size is an early indication of fetus size abnormalities in the BWS and SRS models. In a retrospective human study that included 12 BWS pregnancies, fetal ultrasound detected exomphalos, macroglossia, visceralomgaly, polyhydramnios, and placentomegaly in 67%, 50%, 83%, 58%, and 83% of the cases, respectively, (Kagan et al., 2015). The median gestational age at the time of examination was 22.6 (range, 19.0–29.7) weeks. Aberrant IGF2 levels should be detected at these times according to the corresponding time points during mouse gestation (Figures 4 and 5). Children born by assisted reproductive technologies have a 4- to 10-fold increased frequency of BWS and SRS (Hiura et al., 2012; Mussa et al., 2017; Hattori et al., 2019), and a fraction of these children have a loss of methylation at ICR1. They may also benefit from IGF2 screening. We are optimistic that IGF2 detection will clearly identify the cases that can be helped by adjusting IGF2 signaling in utero.

Which cases would respond to in utero adjustment of IGF2 signaling? Based on the evidence in the mouse rescue models including the current work, we propose that IGF2-based intervention would be successful in each case that displays aberrant IGF2 levels, even though these may have very different molecular features (Figure S1). However, cases with normal IGF2 levels would not respond to IGF2 therapy even if they displayed specific molecular diagnostic features of BWS or SRS disorders, for example those positive for ICR2 epigenotype only. We predict that BWS cases with IGF2 overdose would respond to reducing the IGF2 signaling output and SRS cases with severely reduced IGF2 would benefit from IGF2 enhancing therapy in utero. We speculate that other imprinted disorders may respond to in utero IGF2 treatment if they displayed characteristic fetal growth anomalies and also diagnostic IGF2 levels. One possibility is Temple syndrome (TS14), which has overlapping symptoms with SRS, and TS14 fibroblasts with molecular abnormalities at 14q32.2 displayed low levels of IGF2 RNA expression (Abi Habib et al., 2019).

Our results suggest successful strategies for normalizing IGF2 signaling in utero. IGF2 affects growth by signaling through IGF1R. A BWS case was recently reported with complex genetic abnormalities, paternal uniparental disomy of chromosome 11 (pat(11)dup), and 15q terminal deletion, resulting in two paternal IGF2 copies and also IGF1R haploinsufficiency (Giabicani et al., 2019). The patient exhibited growth retardation after birth but not before birth. This finding is in agreement with the explanation that, as in the mouse, in humans the relative IGF2:IGF1R dose determines growth rate at the fetal stage but not postnatally. We showed that a specific inhibitor of IGF1R kinase activity, PPP (Girnita et al., 2004), reduced IGF2 signaling and normalized the growth in PPP-treated CTCFm/+ fetuses. Their body and internal organs were normal at the time of weaning, suggesting that the BWS-like overgrowth phenotype can be prevented pharmacologically by adjusting IGF2 signaling in utero. PPP affected the CTCFm/+ fetuses to a greater extent than the +/+ fetuses, perhaps because of the increased capacity of the larger CTCFm/+ placenta for transmitting PPP to the fetus. This result is reassuring for human intervention because twinning is relatively more common in BWS than in the general population (Weksberg et al., 2002; Cohen et al., 2019). Other drugs could also be considered, for example a number of different IGF1R targeting drugs that are FDA approved and/or are currently undergoing clinical trials for treating various diseases including cancer and thyroid eye disease (Dolgin, 2020). We have not rescued the SRS model’s growth restriction phenotype pharmacologically, but such treatments are feasible based on large animal studies. IGF1 delivered into the amniotic fluid of intrauterine growth restriction model lambs increased the birth weight (Eremia et al., 2007; Spiroski et al., 2018). Similarly, fetal intra-amniotic IGF2 treatment would benefit human SRS cases that have severely reduced levels of IGF2. It is important to mention that the IGF2:IGF1 ratio is 12:1 in the human amniotic fluid (Merimee et al., 1984; Nonoshita et al., 1994).

We expect that as a consequence of correcting fetal growth and birth weight, other growth-dependent features can also be ameliorated or prevented by correcting IGF2 levels prenatally.
We expect that the rescued newborns would continue to grow normally after birth, as we observed in the mouse rescue experiments. Among BWS symptoms, macroglossia would likely also respond to PPP based on our finding of the partially reduced tongue in weanlings that have undergone PPP treatment in utero. On the other hand, based on our results in the placenta (Figure 6B), we do not expect that placentomegaly could be prevented by PPP. This is consistent with genetic evidence suggesting that IGF1R is not responsible for IGF2 signaling in the placenta (Baker et al., 1993). This is perhaps less of a problem because the placenta is disposed at birth. The association between overgrowth and increased tumor risk in BWS is well documented (Brioude et al., 2019). Wilms tumor and hepatoblastoma display molecular defects consistent with IGF2 overexpression, such as loss of imprinting in the 11p15 region (Honda et al., 2008; Montagna et al., 1994; Rainier et al., 1995; Taniguchi et al., 1995).

Overexpression of IGF2 was also detected in those tumors (Akmal et al., 1995; Scott et al., 1985). The tumor risk in BWS is the highest in patients with molecular defects that predict IGF2 overexpression (28% in patients with ICR1 GOM and 16% in patients with upd(11)pat) but low in patients with other molecular defects (2.6% in patients with ICR2 loss of methylation) (Maas et al., 2016; Mussa et al., 2016). We cannot directly address Wilms tumor and hepatoblastoma because these human complications are not observed in the mouse BWS model. Our results (Figure 6D) predict that kidney size will be corrected in the child after correcting IGF2 signaling activity in the fetus, and this may result in a reduced Wilms tumor incidence. On the other hand, liver size may not be prevented by in utero PPP treatment (Figure 6D).

Pharmacological intervention for IGF2 could be continued postnatally in BWS children and may provide an additional benefit of reducing liver size considering that IGF2 has the longest postnatal presence in the liver. The major IGF2 activity takes place before birth in human children as in the mouse (Constancia et al., 2000; Weber et al., 2001; Lui et al., 2008; also see Figure 5), but the IGF2 mRNA level has a brief postnatal peak at 2 months of age in humans, after which it declines to approximately one-tenth of the peak level (Li et al., 1996). The liver-specific promoter 1 (P1), specifically found in humans, is proposed as a major source of IGF2 (de Pagter-Holthuizen et al., 1998). We expect that an improvement in the BWS and SRS growth symptoms could be achieved by continuing IGF2-based therapies in the first year of life. However, if the correction takes place only after birth, these children will be born with a birth defect.

In summary, our results provide a proof-of-principle for clinical studies that will establish future prenatal diagnosis and treatment options of IGF2-dependent human BWS and SRS.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

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**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2021.108729.

**ACKNOWLEDGMENTS**

We thank Dr. Gerd Pfeifer (VAI) and Dr. Richard Leach (MSU) for helpful discussions, Dr. Timothy Geddes and Samreen Ahmed (Beaumont Hospital, Detroit) for performing the Sequenom allelotyping assays, and the Vivarium at VAI for mouse maintenance and husbandry. The assistance by Yani Sun is greatly appreciated. This work was supported by R01 GM064378/GM/NIGMS NIH HHS/United States to P.E.S. and by VAI.

**AUTHOR CONTRIBUTIONS**

Conceptualization, P.E.S. and J.R.M.; formal analysis, N.P.; funding acquisition, P.E.S.; investigation, J.L., T.-B.Z., N.P., D.A.T., P.S., and P.E.S.; methodology, P.E.S. and J.R.M.; supervision, P.E.S.; validation, J.L., T.-B.Z., N.P., and P.E.S.; visualization, N.P. and P.E.S.; writing—original draft, P.E.S.; writing—review & editing, J.L., T.-B.Z., N.P., D.A.T., P.S., and J.R.M.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

Received: April 25, 2020
Revised: December 2, 2020
Accepted: January 15, 2021
Published: February 9, 2021

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STAR METHODS

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| IGF1 receptor       | Cell Signaling Technology | Cat#3027; RRID:AB_2122378 |
| Phospho-IGF1 receptor (Tyr1135)(DA7A8) | Cell Signaling Technology | Cat#3918; RRID:AB_10548764 |
| AKT (C67E7)         | Cell Signaling Technology | Cat#4691; RRID:AB_915783 |
| Phospho-AKT (Ser473)(D9E) | Cell Signaling Technology | Cat#4060; RRID:AB_2315049 |
| p44/42 MAPK(Erk1/2)(137F5) | Cell Signaling Technology | Cat#4695; RRID:AB_390779 |
| Phospho-p44/42 MAPK (Erk1/2)(Thr202/Tyr204)(D13.14.4E) | Cell Signaling Technology | Cat#4370; RRID:AB_2315112 |
| GAPDH antibody      | ABclonal | Cat#AC035; RRID:AB_2769863 |
| IGF2 antibody       | ABclonal | Cat#A2086; RRID:AB_2764106 |
| Chemicals, peptides, and recombinant proteins | | |
| PPP (AXL1717)       | APExBIO | Cat#A3209 |
| Critical commercial assays | | |
| RNA-Bee reagent     | Tel-Test | Cat#CS-104B |
| Superscript III Random Primer Synthesis kit (Invitrogen\textsuperscript{TM}) | ThermoFisher Scientific | Cat#18080051 |
| DNA-free\textsuperscript{TM} DNA Removal Kit (Ambion\textsuperscript{TM}) | ThermoFisher Scientific | Cat#AM1906 |
| 384 SpectroCHIP Array | Agena Bioscience | Cat#10411 |
| Experimental models: organisms/strains | | |
| Mouse: Rr27tm1Pes (CTCFm) | Szabó et al., 2004 | RRID:MG1:3046755 |
| Mouse: Rr27tm4Pes (CHB) | Lee et al., 2010 | N/A |
| JF1/MsJ inbred strain | The Jackson Laboratory | JAX: 003720; RRID:IMSR_JAX:003720 |
| Oligonucleotides    | | |
| Primers for genotyping, see Table S1 | This paper and Lee et al., 2010 | N/A |
| Primers for RT-PCR, see Table S2 | Lee et al., 2010 | N/A |
| Primers for Sequenom allelotyping, see Table S3 | This paper | N/A |
| Software and algorithms | | |
| SpectroAcquire      | Agena (formerly Sequenom) | N/A |
| MassArray Typer v3.4 | Agena (formerly Sequenom) | N/A |

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Piroska E. Szabó (piroska.szabo@vai.org).

Materials availability
This study did not generate new unique reagents.

Data and code availability
The published article includes all datasets generated or analyzed during this study.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mouse models
All animal experiments were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory animals, with Institutional Care and Use Committee-approved protocols at Van Andel Institute (VAI). We previously generated the BWS
and SRS models using gene targeting in 129S1/ImJ ES cells. The Pgkneo selection cassette was removed by crossing the mutant mice with the Hprt-Cre transgenic mouse line (Tang et al., 2002). The BWS model was created by introducing point mutations in the four CTCF binding sites (CTCFm) in the ICR1 allele (Szabó et al., 2004). To generate the SRS model we replaced the ICR1 with tandem copies of the unmethylated chicken β-globin insulator region (CHB), which contains two functional CTCF binding sites but due to mutagenesis it lacks the boundary elements of the chicken insulator (Lee et al., 2010). Genotyping primers are listed in Table S1. For the present study the CHB mutation was backcrossed ten times into JF1/MsJ inbred strain from The Jackson Laboratory (JAX 003720) to saturate the genome with SNPs, resulting in the CHB.JF1 sub line. Female and male uterus-mate fetuses were used as biological replicates in the experiments that compare RNA levels, allelic expression, IGF2 protein levels or IGF2 signaling between the genotypes. Female and male embryos were combined from multiple pregnancies in experiments to plot growth between genotypes and between treated and control mice. Litter numbers, fetus, newborn, and weanling numbers are provided in the figure legends.

**METHOD DETAILS**

**RNA isolation and quantification**

We purified total RNA using RNA-Beet reagent (Tel-Test). To measure individual transcript levels, we prepared cDNA from 400 ng total RNA for qPCR assays as we did earlier (Lee et al., 2010). Contaminating DNA was removed with the DNA-free DNA Removal Kit (Ambion). Reverse transcription was performed using equal amount of RNA using the Superscript III Random Primer Synthesis kit (Invitrogen). The qRT-PCR primer sequences are listed in Table S2.

**Sequenom allelotyping**

To measure the portion of each parental allele in the total transcript levels we used a multiplex Sequenom (now Agena Bioscience) allelotyping assay as we did earlier (Iqbal et al., 2015). These assays are based on single nucleotide polymorphisms (SNPs) that distinguish between the inbred JF1/MsJ (JF1) and 129S1 (129) mouse strains. Each “unextended” primer (UEP) abuts a SNP in a target transcript, and the incorporating nucleotides differ in molecular mass between the parental alleles. The abundance of the extended UEP is quantified by mass spectrometry. Amplified cDNA samples were spotted onto a 384 SpectroCHIP Array (Agena Bioscience). Automated spectra acquisition was performed in a MassArrayCompact mass spectrometer (Sequenom) using the Spectroacquire program (Sequenom) and was analyzed by MassArray Typer v3.4. RNA-mixing standards were routinely run to verify linear response in measured versus input allele-specific transcription: for example, total RNA from JF1 and 129 embryos was mixed in indifferent percent ratios (0:100, 10:90, 30:70, 50:50,70:30, 90:10, and 100:0) before cDNA preparation and Sequenom allelotyping. A 50:50 RNA mix was used for RNA skew correction. The percentage of transcription of each allele in the total expression was calculated at each given SNP. DNA samples were run from each embryo to verify the heterozygous genotype at each SNP. The unextended extend primer (UEP) and PCR primer sequences are listed in Table S3.

**Western blot**

We obtained amniotic fluid from 13.5 dpc and 16.5 dpc embryos by puncturing the visceral yolk sac and amnion and drawing about 50 µl liquid using a 19-gauge hypodermic needle attached to a 1 ml syringe. The maximum volume of amniotic fluid we harvested depended on the genotype: 50, 75, 75, and 110 µl was drawn from +/-CHB, +/-, CTCFm/CHB, and CTCFm/+ genotypes at 13.5 dpc. We removed the cell content by centrifugation (in an Eppendorf centrifuge, 13000 rpm, 5 min at room temperature). We added equal volume of protein sample buffer (62.5 mM Tris, pH 6.8; 20% glycerol; 2% SDS; 5% 2-mercapto-ethanol; 0.05% Bromophenol Blue) according to the volume of the supernatant we collected. We boiled the sample for 10 minutes, and kept it frozen until electrophoresis. We also collected whole amnion in 50 µl protein sample buffer, whole yolk sac in 200 µl of protein sample buffer and one kidney in 50 µl of protein sample buffer. For detecting IGF2 at 13.5 dpc we used 3 µl of amniotic fluid, 2 µl of amnion, 0.5 µl of yolk sac and 10 µl of kidney protein sample per well for electrophoresis in a 4%–20% gradient protein gel. After blotting, the membranes were incubated with an antibody against IGF2 (ABclonal Cat No A2086 1:1000). The signal was visualized by chemiluminescence and exposure to X-ray film. The images were scanned on a ChemiDoc XRS+ imaging system using Image Lab 5.2.1 (Bio-Rad) software and were quantified using the Fiji software.

For detecting IGF1R and its downstream targets we collected fetal kidney samples at 18.5 dpc. One kidney was homogenized in 200 µl of RIPA buffer supplemented with protease inhibitor and phosphatase inhibitors. The total lysate was left on ice for 30 minutes and sonicated for 5 minutes. After centrifugation for 15 minutes, the supernatant was transferred to a clean tube and the protein concentration was measured by spectroscopy at 280 nm. 10 µg protein was boiled with protein sample buffer and electrophoresed on a 4%–20% gradient SDS-polyacrylamide gel and blotted onto a PVDF membrane. Blots were first reacted with the anti-phosphorylated receptor antibody, then stripped and reacted with the pan-receptor antibody, then stripped again and reacted with the loading control anti-GAPDH antibody. The antibodies used for this experiment were IGF1 receptor (#3027), Phospho-IGF1 receptor (Ytr1135)(DA7A8)(#3918), AKT (C67E7)(#4691), Phospho-AKT (Ser473) (#9272), Phospho-p44/42 MAPK (Erk1/2)(137F5)(#4695), Phospho-p44/42 MAPK (Erk1/2)(Thr202/Tyr204)(D13.14.4E)(#4370). All six antibodies were from Cell Signaling Technology. The GAPDH antibody was from Abclonal (#AC035).
Treatment of mice with PPP
CTCFm/+ females were mated with +/+ males. They were orally dosed daily with 20 mg/kg PPP (AXL1717, APExBIO Cat No A3209) for two days starting at 16.5 dpc, or for five days, starting at 13.5 dpc using cheerio cereal (General Mills) as carrier. In a pilot experiment we used 40 mg/kg PPP for five days and observed a great reduction in the weight of fetuses, even below the untreated normal fetuses. Fetuses and placentas were collected on 18.5 dpc. To control for litter size and for a potential in utero competition between genotypes, we excluded from the analysis the small litters (less than 6 fetuses) and the litters that yielded only one genotype.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical Methods
The normality of distribution within each group of samples was accessed using a Shapiro-Wilk test. If the P-value of the Shapiro-Wilk test was p > 0.05, then the data within the group was determined as normally distributed; whereas a p < 0.05 indicated that the data follow a non-normal distribution. For determining the significance between two normally distributed groups, a non-paired two-sided Student’s t test was used to calculate P-values. When one or both groups followed non-normal distribution, a non-paired two-sided Wilcoxon rank sum test was performed to calculate P-values. Adjusted P-values between two or more normally distributed groups were determined by one-way ANOVA followed by a Tukey post hoc test. For multiple comparisons with at least one non-normally distributed group, Kruskal-Wallis was followed by Dunn’s multiple comparison test to obtain Holm-Bonferroni adjusted P-values. Boxplots and statistics were generated using R v3.6.
Supplemental Information

Prenatal correction of IGF2 to rescue the growth phenotypes in mouse models of Beckwith-Wiedemann and Silver-Russell syndromes

Ji Liao, Tie-Bo Zeng, Nicholas Pierce, Diana A. Tran, Purnima Singh, Jeffrey R. Mann, and Piroska E. Szabó
Fig. S1
Supplemental Figures

Fig. S1 Genetic and epigenetic defects of BWS and SRS at the ICR1 and their mouse models. Related to Figure 1. Based on the insulator model (Fig. 1), the molecular diagnostic features detected at the ICR1 in both BWS and SRS are consistent with misregulation of IGF2 and with aberrant fetal growth.

(A-C) BWS molecular features at the ICR1 predict biallelic IGF2, elevated IGF2 levels and enhanced growth (Azzi et al., 2014, Bachmann et al., 2017, Brioude et al., 2018, Netchine et al., 2013).

(A) Mosaic segmental paternal uniparental isodisomy of chromosome 11p15.5 (upd(11)pat) is found in 20% of BWS cases. Other chromosomal abnormalities of 11p15 are detected in <5% of BWS cases. We previously confirmed the role of IGF2 in the overgrowth phenotype of PatDup.dist7 fetuses that model segmental pat(11)upd after having rescued their lethality using an Ascl2 transgene (ASCL2 is not imprinted in humans) (Rentsendorj et al., 2010).

(B) ICR1 mutations or deletions occur in the maternal chromosome in 1-2% of BWS cases and some of these inactivate the binding sites for the CTCF insulator protein (Riccio et al., 2009). The CTCFm allele used in the current study (Szabó et al., 2004) models this genetic defect.

(C) Gain of methylation (GOM) in the maternal ICR1 is found in 5-10% of BWS cases (Brioude et al., 2018, Brioude et al., 2019). The CTCFm allele used in the current study (Szabó et al., 2004) models the epigenetic defect of ICR1 hypermethylation in the maternal allele.

(D-F) SRS molecular defects at the ICR1 predict reduced IGF2 levels and fetal growth deficit (Azzi et al., 2014, Begemann et al., 2015, Eggermann et al., 2006, Gicquel et al., 2005, Penaherrera et al., 2010, Wakeling et al., 2017).

(D) Mosaic maternal disomy of chromosome 11 or maternal duplication of chromosome 11p15.5 are found in 10% of SRS cases. We earlier rescued the lethality and growth retardation
phenotype in the MatDup.dist7 model mice by genetically normalizing ICR1 insulation and \textit{Igf2} (Han et al., 2010).

(E) A mutation in the \textit{IGF2} gene is inherited from the father in rare cases (Binder et al., 2017, Liu et al., 2017, Yamoto et al., 2017, Begemann et al., 2015). Targeted \textit{Igf2} mutation in the paternal allele (DeChiara et al., 1990, DeChiara et al., 1991) can be considered to be a model for such rare SRS cases. The growth retardation of this model was rescued by a deletion in the maternal allele encompassing the \textit{H19} gene and its 5’ flanking sequence including the ICR1, which allowed \textit{Igf2} expression in the maternal allele (Leighton et al., 1995).

(F) The ICR1 has loss of methylation (LOM) in the paternal allele in up to 60% of SRS cases. This is modeled by the unmethylated CHB allele (Lee et al., 2010) in the current study.
Fig. S2

**A**

Fetus

- CTCFm/+ vs. CTCFm/CHB
- Weight (g)
- P = 3.65E-05

Placenta

- CTCFm/+ vs. CTCFm/CHB
- Weight (g)
- P = 0.031

**B**

- Weight (g) vs. Age (dpp)
- CTCFm/CHB (blue)
- CTCFm/+ (red)
- P = 3.65E-05
- P = 0.031
**Fig. S2. Genetic rescue of the BWS-like overgrowth phenotype. Related to Figure 2.**

CTCFm/CTCFm females were crossed with CHB/+ males to generate offspring with two genotypes, CTCFm/+ and rescued CTCFm/CHB.

(A) Rescue at the fetal stage. Boxplots show the weight distribution of fetuses and placentas at 18.5 dpc by genotype CTCFm/+ and CTCFm/CHB. P-values between the mutant mice compared to the +/+ were calculated using non-paired two-tailed Student’s t-tests.

(B) Postnatal weight gain of individual pups from one large litter is plotted between 1 and 28 dpp.
Fig. S3

Relative Organ Weight

Brain  Heart  Kidney  Liver  Lung  Tongue

Relative Liver Weight

P = 1.06E-12

P = 0.0003

P = 0.006

+++
CTCFm/+
+/CHB
CTCFm/CHB
Fig. S3. Relative organ weights in the disease models and in the compound heterozygote pups. Related to Figure 3. Boxplots show the distribution of relative organ/body weights on the day of weaning. +/CTCFm females were crossed with CHB/+ males to generate offspring with four genotypes. The significant differences between the mutant, and +/+ samples are labeled. 

P-values for the relative brain, heart, kidney, lung, tongue weights were calculated using one-way ANOVA with Tukey post hoc analysis. P-values for the relative liver weights were calculated using Kruskal-Wallis with Dunn’s multiple comparison. The data match the samples displayed in Figure 3A and 3C.
Fig. S4

A

H19/Gapdh

Relative transcript level

B

H19% maternal allele

|   | AM  | BR  | HE  | KI  | LI  | LU  | MU  | PL  | ST  | TO  | YS  |
|---|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| +/+| 94  | 95  | 96  | 75  | 100 | 100 | 96  | 97  | 97  | 97  | 97  |
| +/CHB| 54  | 55  | 55  | 55  | 55  | 55  | 56  | 56  | 56  | 56  | 56  |

Legend:
- Blue: +/+  
- Red: CTCFm/+  
- Yellow: +/CHB  
- Green: CTCFm/CHB

*Relative transcript levels for H19/Gapdh in different samples.*

*H19% maternal allele for different samples.*
Fig. S4. *H19* RNA expression in the fetal organs. Related to Figure 4.

(A) The relative *H19/Gapdh* transcript levels, as measured by real-time reverse-transcription PCR are depicted by bar graphs in 11 organs (amnion, brain, heart, liver, lung, kidney, muscle, placenta, stomach, tongue, and visceral yolk sac) at 18.5 dpc. The average values were plotted with standard deviation, according to the four genotypes. +/CTCFm females were crossed with CHB/+ males to generate these fetuses.

(B) Parental allele-specific transcription of *H19* is shown in the major organs of 18.5 dpc +/+ fetuses and biallelic *H19* transcription is shown in the +/CHB fetuses, as detected by allelotyping. The genotypes are shown to the left. The numbers refer to the percent maternal allele in the total expression determined using multiplex allelotyping assays. 129S1 versus JF1/Ms single nucleotide polymorphisms, inherited from the mothers and fathers were utilized for quantifying parental alleles in the *H19* transcript. The colors depict maternal allele-specific bias in red and biallelic expression in yellow.
|                  | H19 | Igf2 | Igf2as | Acsl2 | Kcnq1 | Kcnq1ot1 | Cdkn1c | Slc22a18 | Phlda2 | Tnfrsf22 | Peg3 | Usp29 |
|------------------|-----|------|--------|-------|-------|----------|--------|----------|--------|----------|------|-------|
| **Amnion**       |     |      |        |       |       |          |        |          |        |          |      |       |
| +/-              | 100 | 0    | 0      | 0     | 0     | 0        | 0      | 0        | 0      | 0        |      |       |
| CTCF/+          | 100 | 0    | 0      | 0     | 0     | 0        | 0      | 0        | 0      | 0        |      |       |
| +/-/CHB         | 100 | 0    | 0      | 0     | 0     | 0        | 0      | 0        | 0      | 0        |      |       |
| **Brain**        |     |      |        |       |       |          |        |          |        |          |      |       |
| +/-              | 100 | 17   | 32     | 0     | 0     | 0        | 0      | 0        | 0      | 0        |      |       |
| CTCF/+          | 100 | 17   | 32     | 0     | 0     | 0        | 0      | 0        | 0      | 0        |      |       |
| +/-/CHB         | 100 | 17   | 32     | 0     | 0     | 0        | 0      | 0        | 0      | 0        |      |       |
| **Heart**        |     |      |        |       |       |          |        |          |        |          |      |       |
| +/-              | 100 | 0    | 0      | 0     | 0     | 0        | 0      | 0        | 0      | 0        |      |       |
| CTCF/+          | 100 | 0    | 0      | 0     | 0     | 0        | 0      | 0        | 0      | 0        |      |       |
| +/-/CHB         | 100 | 0    | 0      | 0     | 0     | 0        | 0      | 0        | 0      | 0        |      |       |
| **Kidney**       |     |      |        |       |       |          |        |          |        |          |      |       |
| +/-              | 100 | 0    | 0      | 0     | 0     | 0        | 0      | 0        | 0      | 0        |      |       |
| CTCF/+          | 100 | 0    | 0      | 0     | 0     | 0        | 0      | 0        | 0      | 0        |      |       |
| +/-/CHB         | 100 | 0    | 0      | 0     | 0     | 0        | 0      | 0        | 0      | 0        |      |       |
| **Liver**        |     |      |        |       |       |          |        |          |        |          |      |       |
| +/-              | 100 | 0    | 0      | 0     | 0     | 0        | 0      | 0        | 0      | 0        |      |       |
| CTCF/+          | 100 | 0    | 0      | 0     | 0     | 0        | 0      | 0        | 0      | 0        |      |       |
| +/-/CHB         | 100 | 0    | 0      | 0     | 0     | 0        | 0      | 0        | 0      | 0        |      |       |
| **Lung**         |     |      |        |       |       |          |        |          |        |          |      |       |
| +/-              | 100 | 0    | 0      | 0     | 0     | 0        | 0      | 0        | 0      | 0        |      |       |
| CTCF/+          | 100 | 0    | 0      | 0     | 0     | 0        | 0      | 0        | 0      | 0        |      |       |
| +/-/CHB         | 100 | 0    | 0      | 0     | 0     | 0        | 0      | 0        | 0      | 0        |      |       |
| **Muscle**       |     |      |        |       |       |          |        |          |        |          |      |       |
| +/-              | 100 | 0    | 0      | 0     | 0     | 0        | 0      | 0        | 0      | 0        |      |       |
| CTCF/+          | 100 | 0    | 0      | 0     | 0     | 0        | 0      | 0        | 0      | 0        |      |       |
| +/-/CHB         | 100 | 0    | 0      | 0     | 0     | 0        | 0      | 0        | 0      | 0        |      |       |
| **Placenta**     |     |      |        |       |       |          |        |          |        |          |      |       |
| +/-              | 100 | 0    | 0      | 0     | 0     | 0        | 0      | 0        | 0      | 0        |      |       |
| CTCF/+          | 100 | 0    | 0      | 0     | 0     | 0        | 0      | 0        | 0      | 0        |      |       |
| +/-/CHB         | 100 | 0    | 0      | 0     | 0     | 0        | 0      | 0        | 0      | 0        |      |       |
| **Stomach**      |     |      |        |       |       |          |        |          |        |          |      |       |
| +/-              | 100 | 0    | 0      | 0     | 0     | 0        | 0      | 0        | 0      | 0        |      |       |
| CTCF/+          | 100 | 0    | 0      | 0     | 0     | 0        | 0      | 0        | 0      | 0        |      |       |
| +/-/CHB         | 100 | 0    | 0      | 0     | 0     | 0        | 0      | 0        | 0      | 0        |      |       |
| **Tongue**       |     |      |        |       |       |          |        |          |        |          |      |       |
| +/-              | 100 | 0    | 0      | 0     | 0     | 0        | 0      | 0        | 0      | 0        |      |       |
| CTCF/+          | 100 | 0    | 0      | 0     | 0     | 0        | 0      | 0        | 0      | 0        |      |       |
| +/-/CHB         | 100 | 0    | 0      | 0     | 0     | 0        | 0      | 0        | 0      | 0        |      |       |
| **Yolk sac**     |     |      |        |       |       |          |        |          |        |          |      |       |
| +/-              | 100 | 0    | 0      | 0     | 0     | 0        | 0      | 0        | 0      | 0        |      |       |
| CTCF/+          | 100 | 0    | 0      | 0     | 0     | 0        | 0      | 0        | 0      | 0        |      |       |
| +/-/CHB         | 100 | 0    | 0      | 0     | 0     | 0        | 0      | 0        | 0      | 0        |      |       |

Fig. S5
Fig. S5. Misexpression of imprinted genes by the CTCFm or CHB mutation is confined to the H19/Igf2 imprinted domain along chromosome 7. Related to Figure 4.

Allele-specific transcription of H19, Igf2 and Igf2as transcripts, together with seven transcripts in the neighboring Kcnq1/Cdkn1c imprinted domain and two distantly located transcripts in chr 7 at 18.5 dpc. Sequenom allelotyping assays of biological duplicate samples are shown. The numbers refer to the percent maternal allele expression in the total. The colors depict maternal allele-specific bias in red, paternal allele-specific bias in blue, and biallelic expression in yellow. The top four samples for each organ are uterus-mates, one female and one male in each genotype. These were obtained by crossing a +/CTCFm (129S1) female with a CHB.JF1/+ (JF1/Ms) male. The bottom four samples for each organ are uterus-mates, one female and one male in each genotype, and were obtained by crossing a +/+ (JF1/Ms) female with a CHB/+ (129S1) male. Note that only H19 Igf2, and Igf2as responded to the ICR1 mutations. Both Igf2 and Igf2as RNAs exhibited paternal expression in the +/+ organs except in the brain, and biallelic expression in CTCFm/+ organs, suggesting that these genes are co-regulated by the ICR1. Also note that there were no other changes in the parental allele-specificity of any other transcripts in the neighboring imprinted domains toward the telomere or upstream toward the centromere. Ascl2 and Kcnq1 transcription is maternal allele-specific in the placenta of +/+ fetuses and also in CTCFm/+ and +/CHB fetuses at 18.5 dpc. Slc22a18 is maternally expressed in the amnion and yolk sac while Phlda2a is maternally expressed in the yolk sac and placenta. Kcnq1ot1, Peg3 and Usp29 are ubiquitously paternally expressed whereas Cdkn1c is maternally expressed in each organ.
| Tissue     | Amnion       | CTCF/CHB     | CTCTm/CHB |
|------------|--------------|--------------|-----------|
| +/+        | +/+          | +/+          | +/+       |
| /+/CHB     | /+           | /+           | /+        |
| /+/CHB     | /+           | /+           | /+        |

| Tissue     | Liver        | CTCF/CHB     | CTCTm/CHB |
|------------|--------------|--------------|-----------|
| +/+        | +/+          | +/+          | +/+       |
| /+/CHB     | /+           | /+           | /+        |
| /+/CHB     | /+           | /+           | /+        |

| Tissue     | Lung         | CTCF/CHB     | CTCTm/CHB |
|------------|--------------|--------------|-----------|
| +/+        | +/+          | +/+          | +/+       |
| /+/CHB     | /+           | /+           | /+        |
| /+/CHB     | /+           | /+           | /+        |

| Tissue     | Muscle       | CTCF/CHB     | CTCTm/CHB |
|------------|--------------|--------------|-----------|
| +/+        | +/+          | +/+          | +/+       |
| /+/CHB     | /+           | /+           | /+        |
| /+/CHB     | /+           | /+           | /+        |

| Tissue     | Placenta     | CTCF/CHB     | CTCTm/CHB |
|------------|--------------|--------------|-----------|
| +/+        | +/+          | +/+          | +/+       |
| /+/CHB     | /+           | /+           | /+        |
| /+/CHB     | /+           | /+           | /+        |

| Tissue     | Stomach      | CTCF/CHB     | CTCTm/CHB |
|------------|--------------|--------------|-----------|
| +/+        | +/+          | +/+          | +/+       |
| /+/CHB     | /+           | /+           | /+        |
| /+/CHB     | /+           | /+           | /+        |

| Tissue     | Tongue       | CTCF/CHB     | CTCTm/CHB |
|------------|--------------|--------------|-----------|
| +/+        | +/+          | +/+          | +/+       |
| /+/CHB     | /+           | /+           | /+        |
| /+/CHB     | /+           | /+           | /+        |

| Tissue     | Yolk sac     | CTCF/CHB     | CTCTm/CHB |
|------------|--------------|--------------|-----------|
| +/+        | +/+          | +/+          | +/+       |
| /+/CHB     | /+           | /+           | /+        |
| /+/CHB     | /+           | /+           | /+        |

Fig. S6
Fig. S6. Allele-specific expression in the imprinted gene network is undisturbed in the insulator mutants and in the compound heterozygotes. Related to Figure 4. Allele-specific transcription was determined for 23 transcripts (marked at the top) in 11 organs using Sequenom allelotyping assays. We crossed +/CTCFm (129S1) females with CHB.JF1/+ (JF1/Ms) males to generate offspring with the four genotypes as indicated to the left. Organs from eight uterus-mate fetuses are shown, one female and one male in each genotype. Note that the allele-specific expression of the genes in the imprinted gene network (Gabory et al., 2009, Monnier et al., 2013) was undisturbed in the insulator mutants and in the compound heterozygotes.
Figure S7

A

Day of pregnancy (dpc)

Weight of mother (g)

Treated 1

Treated 2

Treated 3

Mock 1

Mock 2

B

Number of fetuses per mother

Mother 1

Mother 2

Mother 3

Treated pregnancy

Next pregnancy
Fig. S7. No adverse effect of PPP on pregnant mothers. Related to Figure 6.

The animals were monitored daily. No behavioral or physical abnormalities were observed in the treated mothers. In addition, the PPP (20 mg/kg, five days) treated mothers fed, and groomed the newborns until the weaning day.

(A) Body weights of three CTCFm/+ pregnant females treated with 20 mg/kg dose of PPP for five days, and two mock treated CTCFm/+ females are plotted.

(B) The number of fetuses per mother is tabulated for the first pregnancy (PPP 20 mg/kg for five days) and second pregnancy (no PPP) of three females.
Supplemental Tables

**Table S1. Primers for genotyping. Related to the Star Methods.**

| Primer           | Sequence                      |
|------------------|-------------------------------|
| H19 CTCFmut U2   | GTG GGG TTT ATA CGC GGG AGT TGCATA TGT TTT TCA GCA A |
| H19 CTCFmut L2   | ACT TCA CTG AAA AAC ATA TGC AACTG CCT GGT TTT TAC ACA |
| CTCF1U           | TTG CCG CGT GGT GGC AGC AA     |
| CTCF2L           | ACT TCA CTG CCG TGC G          |
| ChBGL-U          | TGT CTC AGT GTA AAG CCA TTC C   |
| ChBGL-L          | TAA CTT GCT CTT TGT CCT TCT ATC C |

**Table S2. Primers for RT-PCR. Related to the Star Methods.**

| Primer           | Sequence                      |
|------------------|-------------------------------|
| Gapdh exon 5-6 Cy5 | CGTGCCTGGCTGGAGAACCTGCC      |
| Gapdh exon 5-6 U  | AATGTCGTCGTCGATCTCTCT       |
| Gapdh exon 5-6 L  | CAATCTGGCTCCTCAGTGTAGC       |
| Igf2 exon 2-3 HEX | CCTTCAAGCCGTGCAACCAGTCGC    |
| Igf2 exon 2-3 U   | GACCGCGGCTTCTACTTCC         |
| Igf2 exon 2-3 L   | AGCAGCCTCCTCCACGATG        |
| H19 exon 4-5 FAM  | TGCCTACGAAATCTGTCTCAAGGTG   |
| H19 exon 4-5 U    | CTGATCAGAAGATGTCTGCAATC     |
| H19 exon 4-5 L    | GTGTCTATGAGTGCTCTCTCT      |