1   ***Loss of EED in the oocyte causes initial fetal growth restriction followed by placental***

2   ***hyperplasia and offspring overgrowth.***

4   **Short title:** *Epigenetic regulation of fetal growth restriction, placental expansion and*
5   *offspring overgrowth*
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Abstract

Germline epigenetic programming, including genomic imprinting, substantially influences

offspring development. Polycomb Repressive Complex 2 (PRC2) plays an important role in

Histone 3 Lysine 27 trimethylation (H3K27me3)-dependent imprinting, loss of which leads to

placental hyperplasia in mammalian offspring generated by somatic cell nuclear transfer

(SCNT). In this study, we show that offspring from mouse oocytes lacking the Polycomb

protein Embryonic Ectoderm Development (EED) were initially growth restricted,

characterised by low blastocyst cell counts and substantial mid-gestational developmental

delay. However, this initial delay was remediated by striking late-gestational placental

hyperplasia, closely followed by rapid fetal catch-up growth and subsequent overgrowth.

Remodelling of the placenta involved expansion of fetal and maternal tissues, including

conspicuous expansion of the glycogen enriched cell population in the junctional zone.

Genome-wide analyses identified extensive transcriptional dysregulation in affected
placentas, including a range of imprinted and non-imprinted genes. Our data demonstrate
that altered PRC2-dependent programming in the oocyte elicits complex program of fetal
growth restriction followed by placental hyperplasia, which drives catchup growth that
ultimately results in perinatal offspring overgrowth.
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Introduction
Epigenetic mechanisms orchestrate tissue development, body patterning and growth by
regulating chromatin accessibility and expression of developmental genes in embryonic and
extraembryonic tissues (1, 2). Histone 3 Lysine 27 trimethylation (H3K27me3) is a repressive
epigenetic modification catalysed by Polycomb Repressive Complex 2 (PRC2), which is
composed of core components, EED, EZH1/2 and SUZ12, all of which are essential for histone
methyltransferase activity (3-6). Global deletion of any of the individual genes encoding these
PRC2 subunits results in embryonic lethality in mice, while conditional deletions of *Eed, Ezh2*
or *Suz12* result in altered patterning and organ function in a range of tissues (3-5, 7-13). In
humans, *de novo* germline mutations in *EED, EZH2* or *SUZ12* result in Cohen Gibson, Weaver
and Imagawa-Matsumoto syndromes, which all involve overgrowth, skeletal
dysmorphologies and cognitive abnormalities in patients (14-20).
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In mouse oocytes, PRC2 is required for non-canonical H3K27me3-imprinting, whereby
H3K27me3 silences the maternal allele of several genes resulting in paternal allele-specific
expression of the affected genes in pre-implantation embryos and extraembryonic tissues
(21, 22). Oocyte-specific deletion of *Eed* using *Gdf9Cre* resulted in loss of H3K27me3-
imprinting in preimplantation embryos and extraembryonic ectoderm with growth restriction
and male-biased lethality in mid-gestation offspring (23). However, in contrast to growth
restriction, another study that employed a similar strategy using *Zp3Cre* to delete *Eed* in
oocytes found that postnatal offspring were overgrown (7). Moreover, somatic cell nuclear
transfer (SCNT) embryos lack H3K27me3-imprinting, resulting in in bi-allelic expression of
several genes that has been linked to placental hyperplasia and fetal overgrowth(24-27).
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We proposed that loss of EED in the oocyte sets up a developmental trajectory that involves

initial fetal growth restriction that is resolved by placentally driven catch-up growth, which

would explain the apparent stage-dependent discrepancy in the literature. We demonstrate

that while fetuses derived from *Eed* null oocytes were initially growth restricted, they

underwent rapid late-gestational catch-up growth that culminated in postnatal overgrowth.

Detailed analysis of the placenta suggests this complex growth curve is supported by placental

expansion and increased placental efficiency late in gestation. Ultimately, this work reveals a

critical role for EED in the oocyte for regulating a complex program of fetal and placental

growth and function in offspring, and that when EED function is lost in oocytes, placental

hyperplasia drives offspring catchup growth so that growth restriction is ameliorated by

birth.

Results

Loss of maternal EED compromised mid-gestation survival in offspring.

Previous studies demonstrated apparently contradictory findings in that deletion of *Eed* in

oocytes resulted in early embryonic growth restriction (23) and early postnatal offspring

overgrowth (7). To understand how these outcomes are realised, we produced offspring from

pure C57BL6 females producing *Eed*-homozygous null (*hom*), *Eed* heterozygous (*het*) or *Eed*

wild type (*wt*) oocytes and assessed their growth and development throughout the whole

gestational period. Initially, *Eed^{fl/fl}* or *Eed^{fl/wt}* females were mated to males that were

transgenic for *Zp3Cre* to generate females producing *Eed-wt, Eed-het* or *Eed-hom* oocytes (7).

Mating of these females to pure C57BL6 *wt* males allowed us to generate isogenic wild type

or heterozygous offspring as previously described (Fig. 1A) (7). Heterozygous offspring

generated from *Eed* heterozygous oocytes (*HET-het* offspring) or from *Eed* homozygous null

oocytes (*HET-hom* offspring) are isogenic. However, the *HET-hom* offspring were generated

from oocytes that completely lacked functional EED and the *HET-het* offspring were

generated from oocytes that had one functional copy of EED and maintained essentially

normal gene repression (7)(Jarred et al., 2022, submitted). As they are isogenic and

heterozygous, comparison of *HET-hom* with *HET-het* offspring allowed the identification of

differences that resulted specifically from a loss of EED in oocytes in the absence of

confounding genetic differences (Fig. 1A). In addition, we generated wild type offspring from

Eed-het and *Eed-wt* oocytes to compare *WT-wt* and *WT-het* offspring with *HET-het* and *HET-*

hom offspring, providing controls for differences generated as a result of *Eed* heterozygosity

in offspring (Fig. 1A).

Initially, we used automated time-lapse imaging of individual embryos derived from *Eed-wt*

and *Eed-hom* oocytes to track their development from zygote to hatched blastocyst stages.

Embryos from *Eed-hom* oocytes reached the 2-cell stage 1.08h earlier than embryos from

Eed-wt oocytes (Fig. 1B), but blastocyst expansion and hatching took 2.67h longer in *HET-hom*

compared to *WT-wt* embryos (Fig. 1C). All other developmental milestones to blastocyst stage

were similar between genotypes (Fig. 1B). Although, viability to blastocyst stage was lower in

embryos from females producing *Eed-hom* compared to *Eed-wt* oocytes (72.17% vs 88.67%,

Fig. 1D), the proportion of embryos surviving to expanded and hatched blastocyst stages did

not differ significantly (Supp Fig. 1A).

To determine whether the cell content of blastocysts was affected, we performed cell counts

in whole blastocysts and differential staining of inner cell mass (ICM) and trophectoderm (TE)

cells (28)(Supp Fig. 1B). This revealed that overall cell content of embryos from *Eed-hom*

females was lower than in *Eed-wt* controls (Fig. 1E). The number of TE cells was not

statistically altered, but the ICM contained fewer cells in *HET-hom* embryos compared to *WT-*

wt controls (Supp Fig. 1C-D). Together, these data indicated that *HET-hom* embryos contained

fewer cells overall than controls and that the ICM was smaller in embryos from oocytes that

lacked EED compared to oocytes that maintained EED function (Fig. 1F). As previous reports

indicated that maternal deletion of *Eed* did not compromise development to the blastocyst

stage, or cause apoptosis in blastocysts (23), it is likely that lower proliferation explains the

reduction we observed in blastocyst cell number, which affected the ICM rather than the TE.

To assess the effect of oocyte-specific *Eed* deletion on fetal survival and throughout

pregnancy, we compared the number of live fetuses and litter size in offspring generated from

Eed-hom, Eed-het and *Eed-wt* oocytes at multiple stages during gestation and after birth.

Using *Zp3*Cre to delete *Eed* in oocytes, we found no difference between genotypes in the

number of implantations of live embryos in four pregnancies from *Eed*-null oocytes analysed

at E9.5 (Fig. 1G), indicating that *HET-hom* preimplantation embryos implanted at similar rates

and progressed through gastrulation. However, consistent with previous findings (7, 23), the

number of live fetuses at E12.5, E14.5, E17.5, and the number of live born pups were both

significantly lower for *Eed-hom* mothers than for *Eed-het* and *Eed-wt* control females (Fig.

1G). In addition, 71.43% of live fetuses in E17.5 *Eed-hom* pregnancies were female, indicating

male-biased lethality of *HET-hom* offspring (Fig. 1H), consistent with previous studies (23, 29).

Loss of EED in oocytes results in offspring post-implantation developmental delay with

catch-up growth and subsequent postnatal overgrowth.

These observations demonstrated that the major period of fetal loss of *HET-hom* offspring

was between E9.5 and E12.5 in our model or by E6.5 in Inoue, Chen (23), which indicates that

either placental function or embryo survival was compromised in both models. To determine

whether differences occurred in the fetus and/or placenta, we assessed the growth and

development of surviving mid-late gestation offspring. Embryonic day (E)9.5 *HET-hom*

offspring from multiple litters were small compared to *WT-wt* offspring (Fig. 2A). Similarly,

E9.5 *HET-hom* offspring, including extraembryonic tissues, were significantly lighter than *HET-*

het, WT-het and *WT-wt* controls (Supp Fig. 2A). Subsequently, compared to *HET-het, WT-het*

and *WT-wt* controls, *HET-hom* embryos contained fewer tail somites at E12.5 and had

reduced inter-digital tissue regression in foot plates at E14.5 (Fig. 2B, Supp Fig. 2B),

demonstrating that *HET-hom* offspring were developmentally delayed.

This *in utero* developmental delay contrasted markedly with our previous observation that

postnatal *HET-hom* offspring were overgrown at P2 (7). We therefore examined the temporal

developmental trajectory of *HET-hom* offspring by measuring fetal weight at E12.5, E14.5 and

E17.5 and pup weight on the day of birth (P0) and at P3. While weight of *HET-hom* offspring

was significantly lower than *HET-het, WT-het* and *WT-wt* controls at E12.5, E14.5 and E17.5,

by P0 and P3 *HET-hom* offspring were heavier than the isogenic *HET-het* controls (Fig. 2C-G).

Therefore, while *HET-hom* offspring were initially developmentally delayed and under-weight

compared to their isogenic heterozygous counterparts and wild type controls, they

underwent rapid catch-up growth between E17.5 and birth and were overgrown by P3 (Supp

Fig. 2C), consistent with our previous observations at P2 (7).

Oocyte-specific loss of EED results in placental hyperplasia and enhanced placental

efficiency late in gestation which overlaps with *HET-hom* offspring catch-up growth.

To understand whether the catch-up growth observed in *HET-hom* offspring might be related

to changes in placental development, we collected and weighed E12.5, E14.5 and E17.5

placentas from *HET-hom, HET-het, WT-het* and *WT-wt* offspring. At E12.5 placental weights

were consistent across all genotypes, however at E14.5 *HET-hom* placentas were slightly, but

significantly, heavier than *HET-het* controls (Fig. 3A-B). By E17.5 *HET-hom* placentas were 65%

heavier than *HET-het* controls (Fig. 3C), a change reflected in differing *HET-hom* and *HET-het*

placental growth rates over time (Fig 3D-E). While *HET-hom* and *HET-het* placental growth

rates were similar between E12.5 and E14.5, the growth rate of *HET-hom* placentas was 3.29

times greater than that of *HET-het* placentas between E14.5 and E17.5 (Fig 3D-E). Moreover,

consistent with placental expansion driving fetal growth, comparison of placental to fetal

weights revealed that the growth of *HET-hom* placentas immediately preceded the catch-up

growth of late-gestation *HET-hom* fetal offspring (Fig. 3D, F). While fetal growth rate was

initially low between E12.5 and E14.5 and was similar between E14.5 and E17.5 in *HET-hom*

offspring and *HET-het* controls, *HET-hom* offspring gained weight at 1.5 times the rate of *HET-*

het controls between E17.5 and birth (Fig 3F).

To further investigate the relationship between placental function and offspring growth, we

calculated the offspring body to placental weight ratio, which is indicative of placental

efficiency (30). While placental efficiency was consistent across all genotypes at E12.5 (Fig

3G), it was substantially increased at E14.5 and E17.5 in *HET-hom* offspring compared to all

other genotypes (Fig. 3H-I). Histological sections at E17.5 confirmed that *HET-hom* placentas

were obviously larger than *HET-het, WT-het* and *WT-wt* controls. This analysis also revealed

that the junctional zone was expanded in *HET-hom* placentas, with abnormal projections of

periodic acid-Schiff (PAS)-positive spongiotrophoblast cells into the labyrinth (Fig. 3J).

Loss of EED in the oocyte results in altered developmental patterning of the placenta and

extended gestational length.

Given that PAS staining of E17.5 *HET-hom* placenta indicated substantial changes in placental

development, we performed further analyses of *WT-wt*, *WT-het, HET-het* and *HET-hom*

placentas at E14.5 and E17.5 using hematoxylin and eosin (H&E) staining. Consistent with the

modest increase in weight of *HET-hom* compared to *HET-het* placentas at E14.5 (Fig 3B),

quantification of the entire cross-sectional area in H&E-stained sections of E14.5 placentas

demonstrated a 19% increase in *HET-hom* placental area compared to *HET-het* controls (Supp

Fig. 3A & E). This difference was emphasised in overall cross-sectional area in E17.5 *HET-hom*

placentas compared to all other genotypes, with a 55% increase in cross-sectional area

compared to *HET-het* controls (Fig. 4A-B). To understand if this increase in placental size was

due to changes in a specific layer of the placenta, we measured junctional zone, labyrinth and

maternally-derived decidua layer areas. At E14.5, junctional zone area was significantly larger

in *HET-hom* placentas compared to the *WT-wt* and *HET-het* controls, however there was no

difference the *HET-hom* decidua or labyrinth areas compared to all other genotypes (Supp

Fig. 3B-D). At E17.5, the overall cross-sectional area of all layers of *HET-hom* placentas was

greater than that of *HET-het* and *WT-wt* controls (Supp Fig 3E-G). However, as a proportion

of the total placental area, only the junctional zone was significantly greater for *HET-hom*

placentas compared to other genotypes (Fig. 4C-E). Together, these data indicate that the

whole *HET-hom* placenta, including the maternal side, is larger than controls. Moreover, this

is associated with a disproportionate expansion of the fetally-derived junctional zone

between E14.5 and E17.5.

Closer examination of H&E stained E17.5 placentas also showed that there were significantly

more PAS-stained glycogen enriched cells in the junctional zone of *HET-hom* placentas

compared to all other genotypes (Fig. 4F-G). As placental glycogen is converted to glucose to

support fetal growth, the increased glycogen cell number in *HET-hom* placentas is consistent

with a greater glucose requirement in the late gestational fetal catch-up growth observed in

the *HET-hom* offspring. In addition to supporting late-gestational fetal growth, placental

glycogen stores could support the production of hormones that promote parturition towards

the end of the pregnancy (31-34). Consistent with this and the greater numbers of glycogen

enriched cells in *HET-hom* placentas, gestational length was extended by 1 day (7/19 litters)

or 2 days (5/19 litters) in *Eed-hom* pregnancies compared to the *Eed-wt* and *Eed-het* controls

(Fig. 4H). Moreover, in litters where gestational length was extended by one day from 19.5

days to 20.5 days, 36.36% of the *HET-hom* offspring were found dead at birth, and in litters

where gestational length was extended by two days, all *HET-hom* offspring were found dead

at birth. Together, while increased junctional zone area and glycogen cell content

corresponded with fetal catch-up growth, it was also linked to increased gestational length in

almost 2/3 of all litters.

Widespread gene dysregulation occurs in placentas from *Eed-hom* oocytes.

As the developmental changes observed in the placenta indicated that transcriptional regulation may be substantially altered in *HET-hom* placentas we analysed male and female placental tissue from *HET-hom*, *HET-het* and *WT-wt* offspring using RNA-sequencing (RNA-seq). Surprisingly, principal component analysis of the male samples revealed that *HET-hom* placental transcriptomes were indistinct from placental transcriptomes in *HET-het* and *WT-wt* controls (Supp Fig 4A), with only one sequence (CAAA01077340.1) differentially expressed between male *HET-hom* and *HET-het* placentas with an FDR<0.05 (false discovery rate) (Supp Fig 4B,). However, since we observed a male bias in fetal mortality in *HET-hom* offspring, the lack of extensive transcriptional differences between the male placental samples may have been influenced by more moderate effects in surviving males. We therefore focussed our analyses on female placentas only. Principal component analysis revealed that the transcriptome of female *HET-hom* placentas were distinct from their counterpart tissues in *HET-het* and *WT-wt* controls (Fig. 5A). Using an FDR<0.05, 2083 differentially expressed genes (DEGs; together referred to as *Eed* placental DEGs) were identified between *HET-hom* vs *HET-het* placentas (Fig. 5B, Supp Table 1), and only 4 genes (*Eed*, *Fibin*, *Hapln4* & *Dtx1*) were significantly different between *HET-het* and *WT-wt* placentas. This demonstrated that the vast majority of transcriptional dysregulation in the placenta was not due to heterozygosity of *Eed* but can be best explained by non-genetic effects caused by loss of EED specifically in the oocyte. Gene ontology analysis indicated that the *Eed* placental DEGs are involved in morphogenesis, system development and multicellular organism development (Fig. 5C), suggesting that the gene dysregulation observed in the *HET-hom* placenta influences a diverse range of systems and processes.

As sequencing was performed on the entire placenta, we compared the *Eed* placental DEG list with published single cell RNA-seq data from E14.5 C57BL/6 placentas as an indication of the placental cell types in which gene dysregulation occurred in our model (35). Although the E14.5 placental stage assessed by Han, Wang (35) was earlier than the E17.5 placental data collected in our analysis, the majority of mature placental cell types are established by E14.5 (36). We therefore considered the Han, Wang (35) data useful for predicting the spatial expression for our *Eed* placental DEGs. Of the 2083 *Eed* placental DEGs identified in the *Eed*

HET-hom placentas, 543 genes were found in the E14.5 mouse placental data set (35). Cell-
specific comparisons revealed that of these 543 genes, 166 *Eed* placental DEGs included genes
preferentially expressed in the junctional zone and 57 *Eed* placental DEGs were preferentially
transcribed in the labyrinth, which we defined as '*Eed* junctional zone DEGs' and '*Eed*
labyrinth DEGs', respectively (Fig. 5D-E). In addition, 104 *Eed* placental DEGs included genes
preferentially transcribed in the maternally-derived decidua, which we refer to as '*Eed*
decidua DEGs' (Fig. 5F). Together these data revealed that gene dysregulation in *HET-hom*
placentas was not isolated to a single region, and that both maternal and fetal-derived cells
were altered. Of the *Eed* junctional zone DEGs the majority (86.1%) were upregulated,
whereas a large proportion of *Eed* labyrinth DEGs and *Eed* decidua DEGs were downregulated
(86% and 97.1%, respectively; Fig. 5G-I). Together these data demonstrate a strong bias for
up-regulation of genes expressed in the junctional zone layer of the *Eed* placenta. This reflects
the significant expansion of the junctional zone as a proportion of the total placental size.
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Eed placental DEGs included both H3K27me3, classically imprinted genes and some X-linked
genes.
Given the loss of H3K27me3-dependent imprinting at specific genes leads to placental
hyperplasia in Somatic Cell Nuclear Transfer (SCNT) embryos we investigated whether
imprinted genes were dysregulated in the *Eed HET-hom* placenta. This revealed that of 325
known or proposed classically imprinted genes (37, 38), 46 were *Eed* placental DEGs (Fig. 6A,
Supp Table 2). Of 76 putative non-canonical H3K27me3 imprinted genes identified by Inoue,
Jiang (22), 16 were dysregulated in *HET-hom* placenta (Fig. 6A, Supp Table 1). These included,
Slc38a4, *Sfmbt2*, *Gab1* and *Smoc1*, for which loss of H3K27me3-dependent imprinting results
in placental hyperplasia and/or defective offspring development in SCNT-derived offspring
(25-27). Moreover, *Slc38a4, Sfmbt2, Gab1 and Smoc1* were all up-regulated in *HET-hom*
placenta, suggesting a loss of H3K27me3-imprinting and abnormal expression of the maternal
allele of these genes (Fig. 6B). As a range of classically imprinted genes are also aberrantly
expressed in placental cells of SCNT-derived pregnancies (27), we also determined the overlap
of *Eed* placental DEGs with imprinted genes that were dysregulated in SCNT placentas. Of the
325 classically imprinted genes(37, 38), nine were commonly dysregulated (5 up-regulated
and 4 down-regulated) in *HET-hom* placentas and SCNT derived placental cells. In addition,

the H3K27me3-imprinted genes *Sfmbt2*, *Slc38a4*, *Smoc1* and *Gab1* were all upregulated in

Eed HET-hom placentas, but only *Sfmbt2* dysregulated in placentas of SCNT-derived offspring.

As maternal EED is also required for regulating X-inactivation in pre-implantation embryos,

and loss of EED in oocytes caused a male bias in fetal lethality in *Eed HET-hom* offspring (Fig.

1H)(23, 29), dysregulated X-inactivation may provide another explanation for the gene

dysregulation detected in *Eed HET-hom* placenta. To determine if gene expression from the

X-chromosome was preferentially impacted in *HET-hom* placentas, we examined the

representation of the placental DEGs on all autosomes and the X-chromosome. Placental

DEGs with increased expression occurred at a similar rate on the X-chromosome and

autosomes, indicating that there was no preferential chromosome-wide de-repression of X-

linked genes (Fig. 6C). However, we did note upregulation of X-linked genes *Plac1* and *Ldoc1,*

and the imprinted genes *Ascl2* and *Peg3*, which are all known to regulate placental glycogen

stores (34) (Supp Fig. 5A). All were increased in the junctional zone DEG list and have been

associated with similar placental phenotypes to that observed in here *Eed Het-hom* placentas

(Supp Fig. 5A) (39-44). In line with its increased transcription, PLAC1 protein was also

increased in cells within the junctional zone (Fig. 6D).

Finally, we compared *HET-hom* growth patterns with available weight data reported for

SCNT-derived offspring, in which H3K27me3 imprinting is disrupted (27). As for *Eed HET-hom*

offspring, SCNT-derived offspring were underweight at E14.5, but had caught up by birth

(Supp Fig. 6A-B), demonstrating that the growth profiles and placental phenotypes in these

models are similar.

Discussion

Independent studies have demonstrated that deletion of *Eed* in mouse oocytes using *Zp3Cre*

or *Gdf9Cre* results in early growth restriction and offspring overgrowth outcomes in C57BL6

mice (7, 23). This study provides a cohesive explanation for these differing reports. We

observed time-dependent and inter-related fetal and placental growth trajectories of

offspring from *Eed-null* oocytes. Deletion of *Eed* in the oocyte initially led to embryonic and

fetal developmental delay, followed by pronounced placental expansion and subsequent

rapid catch-up and over-growth of the offspring realised in the early postnatal period.

Fetal growth restriction is commonly caused by placental insufficiency and is often investigated using surgical or nutritional interventions in animal models (45-49). In this study we provide an intervention that involves only the deletion of *Eed* in the oocyte and in which *HET-hom* offspring were compared to genetically identical *HET-het* controls. We detail fetal growth restriction immediately prior to placental expansion, indicating a role for the placenta in responding to fetal growth restriction to drive offspring catch-up growth prior to birth. Importantly, placental efficiency increased late in gestation and placental hyperplasia immediately preceded the catch-up growth observed in *HET-hom* offspring. Similar outcomes were common between at least two models – the *Eed* oocyte deletion model described here and an SCNT model in which we found published data indicating similar growth restriction occurred prior to placental hyperplasia and subsequent fetal catchup growth was evident. Therefore, in both models, and potentially in others, the data support a model in which initial growth restriction can be corrected by placental expansion at later stages. An imperative for offspring survival is for the delivery of sufficiently developed offspring. Considering this, an interesting conjecture may be that an unknown physiological mechanism detects fetal growth restriction, causing the placenta to respond and mediate intrauterine catch-up growth, potentially rescuing the pregnancy. While speculative, such a mechanism could involve epigenetic adaptation, perhaps H3K27me3-dependent or classical imprinting.

We observed that the fetal growth restriction, placental hyperplasia and subsequent offspring overgrowth in *Eed HET-hom* offspring was similar to that evident in SCNT-derived offspring. This indicates that the growth patterns for offspring from *Eed*-null oocytes and SCNT are similar. While both models involve loss of H3K27me3-dependent imprinting (23, 26, 27), the SCNT embryos are different to offspring from *Eed*-null oocytes because they were derived from oocytes containing functional maternal PRC2. This might indicate that altered H3K27me3-dependent epigenetic patterning of the oocyte genome is responsible for the early developmental delay in these offspring. However, the underlying cause could differ. SCNT embryos are derived by oocyte-driven reprogramming of a differentiated somatic nucleus, a process through which major epigenetic barriers must be overcome and which may compromise preimplantation development, particularly given that the embryos are transferred to recipient female mice (26, 50-52). Similarly, either loss of maternal PRC2 or

H3K27me3 programming caused by *Eed* deletion in the oocyte could explain the reduced

blastocyst cell content, and this may underlie early embryo and fetal growth restriction. One

way to separate these issues may be to perform pronuclear transfer from *Eed-null* oocytes to

wild type oocytes to rescue maternal PRC2 in offspring, and vice versa.

Comparison of isogenic *HET-hom* and *HET-het* offspring demonstrated that the phenotypic

outcomes in this model originate from loss of EED in the oocyte and were largely unaffected

by offspring genotype. While *HET-het* offspring maintained normal fetal growth, placental

development, gestational length, and litter size, all these aspects of development were

substantially modified in isogenic *HET-hom* controls. Other studies have reported that pre-

implantation development proceeds normally in offspring from *Eed-null* oocytes, in that

blastocysts formed at similar rates and were not affected by increased cell death (23).

However, we found that blastocysts derived from *Eed-null* oocytes contained low cell

numbers, and that this primarily affected the inner cell mass. Consistent with this, we

observed substantial developmental delay in E9.5-E17.5 *HET-hom* offspring derived from *Eed-*

hom oocytes, but this was resolved by birth and perinatal offspring were ultimately larger

than controls.

There are at least two likely explanations for the early developmental delay observed in this

model. EED supplied in the mature oocyte is rapidly localised to the maternal and paternal

pronuclei within the first few hours following fertilisation (23, 29, 53, 54) (Jarred et al 2022,

submitted). As the *HET-hom* offspring derived from *Eed-null* oocytes lacked maternal EED, it

is possible that the lower cell numbers observed at the blastocyst stage resulted from low cell

proliferation during pre-implantation development due to a lack of maternally-derived PRC2.

This is consistent with the established role for PRC2 in driving cell division in stem cells and

other cell types (55) and with a previous report that apoptosis was not increased in blastocysts

from EED-null oocytes (23). Alternatively, the lower blastocyst cell counts we observed may

have resulted from loss of EED-dependent epigenetic programming in growing oocytes. This

programming occurs during a transient stage of PRC2 expression in primary-secondary stage

follicles prior to the onset of DNA methylation (22, 56) (Jarred et al 2022, submitted).

We observed widespread morphological changes in *HET-hom* placental tissue, which
presumably provides the required support for late-gestational offspring catch-up growth.
Importantly, placental expansion was observed in both fetally- and maternally-derived
placental layers, indicating that while placental hyperplasia may be driven through the
spongiotrophoblast cells in the junctional layer, the maternal side of the placenta also
expands to accommodate greater placental function. Despite this, there was a
disproportionate increase in size of the fetally-derived junctional zone compared to the
maternally-derived decidual layer. Furthermore, closer examination of the enlarged
junctional zone revealed an increase in glycogen enriched cells. This was unusual as glycogen-
enriched trophoblasts peak in number at E16.5, but then decline by approximately 60% by
E18.5 (34), an outcome reflected in the decreased placental weights we observed in E17.5
control offspring. It seems likely that the sustained high levels of glycogen stores in the E17.5
placentas of *HET-hom* offspring increase glycogen release to the embryo and drive rapid
growth catch-up growth. Glycogen stores in placenta have been proposed to drive fetal
growth in other models, however a role for placental conversion of glycogen to glucose for
driving fetal catch-up growth is yet to be definitively demonstrated (34). Such a mechanism
may act in concert with increased amino acid transport potentially driven by *Slc38a4*, an
observation made in SCNT-derived placental hyperplasia (27).

While fetal growth catch-up is likely to be beneficial for the chances of successful delivery of
live offspring, there may be a price to pay for this developmental change in the placenta. In
addition to facilitating increased glucose release and rapid fetal catchup growth, glycogen-
producing trophoblasts are also considered to facilitate hormonal release from the placenta
to the maternal blood supply to prepare the mother for parturition (34, 39). It may be that
the increased number of glycogen cells remaining in the late-gestation *HET-hom* placenta
delays late gestational hormonal release from the placenta and prolongs pregnancy.
Consistent with this, extended gestational length occurred in about two thirds of the
pregnancies from *Eed-hom* oocytes. Moreover, while all pups were naturally born, with no
apparent birthing difficulties for the mothers, the extended gestational length observed was
also associated with very high pup mortality in pregnancies extended by one day and
mortality of all offspring in pregnancies extended by two days. While the mechanisms

underlying this extended gestation remain obscure, they may have broader implications for
understanding the impacts of fetal growth changes on pregnancy outcomes.

In addition to the morphological alterations observed, we found extensive transcriptional
dysregulation in *HET-hom* placentas, with 2083 genes differentially expressed compared to
genetically identical *HET-het* placentas. This altered gene expression was not isolated to a
single region of the *HET-hom* placenta and was observed in both fetal and maternal layers.
While one might expect oocyte-specific loss of EED to affect only fetally-derived placental
tissue, this ignores the possibility that the maternal tissue may expand or be re-modelled in
response to placental hyperplasia in fetally-derived layers – indeed, without maternal re-
modelling it seems likely that the placenta would not properly increase function and support
fetal catch-up growth. On this basis, it is unsurprising that transcription was substantially
altered in both fetally- and maternally-derived placental tissues.

Analyses of *HET-hom* placentas identified cellular and morphological changes in maternally-
and fetally-derived tissues and placental DEGs that function in multiple systems. Recent work
has demonstrated that loss of H3K27me3-dependent imprinting at *Slc38a4* or *Sfmbt2,* and to
a lesser extent, *Gab1* or *Smoc1,* leads to placental hyperplasia in SCNT-derived offspring (25-
27, 57). Consistent with this, we observed placental hyperplasia and increased expression of
Slc38a4, Sfmbt2, Gab1 and Smoc1 in placentas from *Eed-null* oocytes, suggesting a loss of
H3K27me3-dependent imprinting at these genes. We also observed increased placental
expression of the X-linked genes *Plac1*, *Wdr1* and *Ldoc1,* and immunohistochemistry
confirmed that PLAC1 expression was increased in junctional zone cells in *HET-hom* placentas.
However, deletion of *Plac1*, rather than increased expression, has been associated with a
similar placental phenotype and male biased lethality to that observed in *HET-hom* offspring
(58). Notwithstanding this difference, it seems likely that the fetal growth restriction,
placental hyperplasia, and fetal catch-up growth in this model involves one or all of these
genes. Substantial additional work is required to understand the mechanisms involved and
the interactions between the genes that contribute.

While we observed no difference in the number of surviving hatched or expanded blastocysts
from *Eed-hom* mothers, there was a decrease in the blastocyst cell count, particularly in the

ICM, and a marked decrease in *HET-hom* litter size. Consistent with this and with another study (23), we also observed smaller offspring at E9.5, developmental delay and loss of embryos by E12.5, and reduced litter size at term (7). While the reason for *HET-hom* offspring loss is yet to be identified, one possibility is that the significant reduction in blastocyst cell numbers and subsequent developmental delay is too profound to support survival during mid-gestation development in the most highly impacted offspring. This may involve impacts within the embryo itself, and/or contributions from a defective placenta. The placenta develops a capacity to exchange nutrients, other supportive metabolites, and waste between the maternal and fetal blood supply at E9.5, and increased embryo loss around this stage could indicate early-mid gestational placental insufficiency (59, 60). Embryonic lethality has also been previously linked to placental defects in knockout studies of imprinted and X-linked genes *Plac1*, *Ascl2* and *Peg10* (39-41, 43, 44, 61). As *Plac1* and *Ascl2* transcription was increased in the late-gestation *HET-hom* placenta, altered expression of imprinted genes such as *Plac1* or *Ascl2* may contribute to placental defects and fetal death in this model.

In summary, we demonstrate that loss of PRC2 function in the oocyte profoundly affects growth and developmental outcomes in both offspring and placenta via a mechanism that is independent of genetic background. This involves initial embryonic developmental delay and fetal growth restriction, followed by remarkable placental expansion, increased placental glycogen stores and fetal catch-up growth (Fig 7). These outcomes were associated with increased expression of genes associated with placental hyperplasia. Importantly, rapid fetal growth restriction and fetal catch-up growth have been linked with negative health outcomes later in life, including metabolic conditions (62, 63). Moreover, given that mutations in the core PRC2 genes, EED, EZH2 and SUZ12 have all been associated with overgrowth and a range of co-morbidities in Cohen-Gibson, Weaver and Imagawa-Matsumoto syndrome patients (14, 15, 17, 19, 20) and we have observed similarities in outcomes of mouse offspring lacking EED in oocytes (7) this work also has implications for understanding these rare conditions. Together, this research reveals that altered PRC2-dependent programming in the oocyte elicits a complex intrauterine environment for the fetus, which has the potential to mediate impacts on offspring health and disease that may persist into adulthood.

Methods

Mouse strains, animal care and ethics

Mice were housed using a 12h light-dark cycle at Monash Medical Centre Animal Facility, as previously reported (7). Room temperature was maintained at 21-23°C with controlled humidity, and food and water were provided *ad libitum*. All animal work was undertaken in accordance with Monash University Animal Ethics Committee (AEC) approval. Mice were obtained from the following sources: *Zp3Cre* mice (C57BL/6-Tg 93knw/J; Jackson Labs line 003651, constructed and shared by Prof Barbara Knowles(64), *Eed* floxed mice (*Eed*^{fl/fl}) (B6; 129S1-*Eed*tm1Sho/J; Jackson Labs line 0022727; constructed and shared by Prof Stuart Orkin (65). The *Eed* line was backcrossed to a pure C57BL6/J and shared with us by Associate Professor Rhys Allen and Professor Marnie Blewitt, Walter and Eliza Hall Institute for Medical Research, Melbourne.

Genotyping

Genotyping was performed by Transnetyx (Cordova, TN) using real-time PCR assays (details available upon request) designed for each gene as described previously (7).

Collection and culture of pre-implantation embryos

Eight to twelve-week-old female mice were superovulated and mated to C57BL/6 males for one night. Zygotes were collected in handling media (G-MOPS PLUS, Vitrolife) at 37°C (28, 66) denuded of cumulus cells with G-MOPS PLUS containing hyaluronidase. All embryos were washed in G-MOPS PLUS and embryo development kinetics was assessed using the EmbryoScope™ (Vitrolife) time-lapse imaging system. Embryos were cultured individually in 25µl of medium, with time-lapse images generated at 15-minute (min) intervals throughout the culture period.

Cell allocation in blastocysts

Following EmbryoScope™ culture, differential staining was performed (67) in hatched blastocysts using propidium iodide to label TE nuclei, while leaving the ICM unlabelled. After fixation, embryos were treated with bisbenzimide to stain ICM and TE, whole-mounted in glycerol and imaged using an inverted fluorescence microscope (Nikon Eclipse TS100). Nuclei were counted using ImageJ.

Collection of post-implantation embryos, placenta and postnatal offspring

Mice were time mated for two-four nights, with females plug checked daily. Positive plugs were noted as day E0.5. Pregnant females were euthanised and embryos collected at E9.5, E12.5 E14.5 and E17.5. E9.5 whole sacs were weighed, fixed in 4% PFA for 72h at 4°C degrees processed, paraffin embedded, sectioned at 5μm. Whole-mount images of E9.5 embryos were taken using a LEICA M80 light microscope with LEICA MC170 HD camera attachment. E12.5, E14.5 and E17.5 embryos and placentas were isolated and weighed. Placentas were then bisected, and half was fixed in 4% PFA for 72h at 4°C degrees, processed and paraffin embedded. The other half of the placenta was rinsed in PBS, snap-frozen on dry ice and stored at -80°C for RNA analysis. P0 and P3 offspring were weighed and euthanized by decapitation. All offspring samples were collected from at least three different females / genotype.

Placental Histology

Paraffin embedded placentas were sectioned at 5μm using a Leica microtome and sections transferred to Superfrost plus slides (Thermo-Fisher, Braunschweig, Germany). Periodic antigen-shiff (PAS) and hematoxylin and eosin (H&E) staining was performed on placental sections by the Monash Histology Platform (MHTP node). Stained slides were scanned using Aperio slide scanner by the Monash Histology Platform and analysis was conducted using QuPath v0.2.3[42]. Junctional zone, labyrinth and decidual area was calculated in E14.5 and E17.5 H&E stained placentas. Glycogen cell counts were also conducted on H&E stained E14.5 and E17.5 placentas using QuPath v0.2.3[42]. Investigators were blinded for sample genotypes throughout quantitative scoring of placental samples analysed.

Immunohistochemistry

Slides with 5μm thick placental sections were baked at 60°C for 20 mins. Tissue sections were dewaxed in three changes of xylene and rehydrated in three changes of ethanol then rinsed in distilled water. Antigen retrieval was performed in DAKO PT Link in a DAKO Target Retrieval (Low pH) Solution (DAKO, Cat# S1699) at 98°C for 30 min. Slides were then washed in DAKO EnVision Flex Wash Buffer (Cat# K8000) for 5 minutes. IHC was then performed on a DAKO Autostainer Plus in the following steps. Sections were washed once in EnVision Flex Wash

Buffer following each subsequent step. Peroxidase Blocking Solution (DAKO, Cat# S2023) was applied for 10 minutes and non-specific binding was prevented with AffiniPure Fab Fragment Goat Anti-Mouse IgG for 1 hour. Mouse anti-PLAC1 (G-1) (Santa Cruz, Cat# sc-365919) primary antibody was applied at an appropriate concentration for 1 hour. EnVision System-HRP Labelled Polymer Anti-Mouse (DAKO, Cat# K4001) was applied for 1 hour. Immunostaining was visualised using DAKO Liquid DAB+ Substrate Chromogen System (Cat# K3468). A counterstain DAKO Automation Haematoxylin Staining Reagent was then applied for 10 minutes. Slides were removed from the Autostainer, transferred to a slide staining rack and rinsed in distilled water. In a fume-hood, slides were then washed in Scott's Tap water and distilled water. Finally, slides were dehydrated in three changes of 100% Ethanol, cleared in three changes of Xylene and mounted in DPX. Slides were either scanned using a VS120 Slidescanner (Olympus).

Placental RNA-sequencing

Placental isolation is described above, and placental RNA was extracted from 4-5 samples/genotype using NucleoSpin RNA Plus columns. RNA quality was assessed on an Agilent Bioanalyser and samples with RIN >7.5 used for library preparation and sequencing on the BGI Genomics platform (BGI Genomics, Hong Kong).

Statistical Analyses

Statistical analyses were performed using GraphPad Prism according to the statistical triage provided in the package. In brief, for parametric tests one way ANOVA with multiple comparison testing was used, with Student's *t* tests for pairwise comparisons included. If indicated in GraphPad Prism, non-parametric were applied. Tests used are indicated in figure legends.

RNA-sequencing data analyses

Adaptor and low-quality sequences in raw sequencing reads were trimmed using Trimmomatic (68) (v0.39) with the following parameters: LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:20. Clean reads were mapped to the mouse reference genome (GRCm38) using STAR (v2.7.5c) with the following settings: outFilterMismatchNoverLmax 0.03 --alignIntronMax 10000. Raw counts for mouse reference

genes (ensembl-release-101) were calculated using STAR (v2.7.5c) with parameter "--
quantMode GeneCounts" simultaneously when doing the genome mapping. Differential gene
expression analysis was carried out using the R package "limma" (69) with "treat" function
and parameter "lfc=log(1.1)". Statistically significantly differentially expressed genes were
identified using "FDR < 0.05". Gene Ontology (GO) enrichment analysis for significantly
differentially expressed genes was carried out using The Database for Annotation,
Visualisation and Integrated Discovery (DAVID) with following settings: GO term level 3,
minimum gene count 5, and FDR < 0.05 (70).
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587 **Declarations**
588
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594
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596 All animal work was undertaken in accordance with Monash University Animal Ethics
597 Committee (AEC) approval.
598
599 **Availability of data and materials**
600 All RNA sequencing data have been deposited to the Gene Expression Omnibus (GEO) and are
601 publicly available with accession number GSE210398. All other information is available from
602 the corresponding author.
603
604 **Competing interests:**
605 The authors declare that they have no competing interests that affect this work
606
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Conceived and/or designed experiments: RO, SP, TT, ZQ, EJ, HB, PSW
Performed experiments and/or analysed data: RO, SP, TT, TTT, DF, ZQ, EJ, HB, PSW
Bioinformatic analyses and/or comparisons with published datasets: ZQ, RO, SP, DLA, PSW
Resources and/or supervision PSW DKG, MvdB, DLA, NAS
Writing - original draft: RO, SP, PSW

Writing - review & editing: RO, SP, EJ, PSW, NAS. All authors critically read and approved the
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Figure 1

 Figure Legends

Figure 1. Maternal *Eed* deletion impacted pre-implantation development. (a) Model used to

generate heterozygous isogenic offspring from oocytes that lacked or retained *Eed.* Females

producing *Eed-wt*, *Eed-het* and *Eed-hom* oocytes were mated with wild type males to produce

offspring from oocytes with *wt* or *het* EED-dependent programming or from oocytes that lack

EED-dependent programming respectively. *Eed-wt* oocytes produce *WT* offspring (*WT-wt*

control), *Eed-het* oocytes produce *WT* or *HET* offspring (*WT-het* and *HET-het*) from the same

GV oocyte, and *Eed-hom* oocytes produce *HET* offspring (*HET-hom*). *HET-het* and *HET-hom*

offspring are isogenic, but were derived from oocytes that had different EED-dependent

programming. **(b)** Cell cleavage and development times of embryos from *Eed-wt* or *Eed-hom*

oocytes in *ex vivo* culture. Data is presented as time to reach 2-, 4-, 8-cell, morula (M),

blastocyst (B), expanded blastocyst (EB) and hatched blastocyst (HB). *$P<0.05$, two-tailed

student's t test, N = 49 embryos from *Eed-wt* oocytes, 57 embryos from *Eed-hom* oocytes. **(c)**

Time taken for expanded blastocysts to hatch. Data represents the time difference between

hatched blastocyst and expanded blastocysts. *$P<0.05$, N = 38 embryos from *Eed-wt* oocytes,

31 embryos from *Eed-hom* oocytes. **(d)** Proportion of embryos from *Eed-wt* or *Eed-hom*

oocytes surviving to blastocyst. *$P<0.05$, Data represent the proportion of surviving embryos

from each female, N = 6 females. **(e)** Total number of cells per embryo. **$P<0.005$, N = 17

embryos from *Eed-wt* oocytes, 30 embryos from *Eed-hom* oocytes. **(f)** Proportion of TE and

ICM cells per embryo. Data represents the mean proportion of cells allocated to TE versus

ICM for embryos from *Eed-wt* or *Eed-hom* oocytes. **$P<0.05$, N = 17 embryos from *Eed-wt*

oocytes, 28 embryos from *Eed-hom* oocytes. **(g)** Offspring litter size over time. ****$P<0.0001$.

(h) Pie chart depicting the proportion of male and female *HET-hom* offspring at E17.5. N=32.

For **(b-g)** a two-tailed student's t test was used and error bars represent mean ± SD.

Figure 2

Figure 2. Loss of EED-dependent oocyte programming resulted in fetal loss and offspring

developmental delay. (a) Representative wholemount images of E9.5 embryos. **(b)** Number

of tail somites in E12.5 *WT-wt, WT-het*, *HET-het* and *HET-hom* embryos. ****P<0.0001,

***P<0.0005, N=10-23/genotype. **(c-g)** Offspring body weight at E12.5, E14.5, E17.5 and P0.

****P<0.0001, ***P<0.0005, **P<0.005, N=15-75/genotype. **(b-g)** statistically analysed using

one-way ANOVA plus Tukey's multiple comparisons.

A   E12.5 Placenta Weight

Weight (g)

Offspring: WT / WT / HET / HET
Oocyte: wt / het / het / hom

B   E14.5 Placenta Weight

Weight (g)

*

Offspring: WT / WT / HET / HET
Oocyte: wt / het / het / hom

C   E17.5 Placenta Weight

Weight (g)

****
****
****
*
*

Offspring: WT / WT / HET / HET
Oocyte: wt / het / het / hom

D   Offspring and Placenta Weight Over Time

— HET-het
— HET-hom

Catch-up growth window

Body weight (g)

*** E12.5   **** E14.5   ** E17.5   *** P0   **** P3

Placenta weight (g)

* E12.5   * E14.5   **** E17.5   P0   P0

E   Placental growth rate (weight change/time)

	HET-het	*HET-hom*
E12.5-E14.5	0.00921	0.00845
E14.5-E17.5	-0.00522	0.0119667

F   Offspring growth rate (weight change/time)

	HET-het	*HET-hom*
E12.5-E14.5	0.08191	0.058235
E14.5-E17.5	0.2183333	0.2079
E17.5-E19.5/P0	0.18615	0.27475

G   E12.5 Placental Efficiency

Body weight/placenta weight (g)

Offspring: WT / WT / HET / HET
Oocyte: wt / het / het / hom

H   E14.5 Placental Efficiency

Body weight/placenta weight (g)

****
**
****

Offspring: WT / WT / HET / HET
Oocyte: wt / het / het / hom

I   E17.5 Placental Efficiency

Body weight/placenta weight (g)

****
****
****

Offspring: WT / WT / HET / HET
Oocyte: wt / het / het / hom

J   E17.5 Periodic acid-Schiff (PAS)

Offspring: WT / WT / HET / HET
Oocyte: wt / het / het / hom

Figure 3

Figure 3. Placental differences were observed in heterozygous offspring that

lacked *Eed*. (a-c) Placental weight at E12.5, E14.5 and E17.5 ****P<0.0001, *P<0.05, N=32-

68/genotype. **(d)** Isogenic heterozygous offspring and placenta average weight over time.

*P<0.05, **P<0.005, ***P<0.0005, ****P<0.0001 unpaired t-test, N=28-56/genotype. **(e-f)**

Tables depicting *HET-het* and *HET-hom* offspring and placenta growth rates across 2-3 time

points throughout gestation. **(g-i)** Ratio of placental weight to offspring body weight at E12.5,

E14.5 and E17.5. ****P<0.0001, **P<0.005, N=9-66/genotype. **(j)** Placental cross-sections at

E17.5 stained with PAS. Scale bars= 800μm. **(a-c, g-i)** statistically analysed using one-way

ANOVA plus Tukey's multiple comparisons.

Figure 4

Figure 4. Complete deletion of *Eed* in the oocyte resulted in offspring altered placental

morphology and gestational length. (a) Placental cross-sections at E17.5 stained with H&E,

black dotted line indicates the borders between labyrinth, junctional zone and decidual

layers. Images are representative of an individual placenta typical of each genotype, with 10

biological replicates analysed/genotype. Scale bars= 500μm. **(b)** Placental cross-sectional area

at E17.5. ***$P < 0.0005$, ****$P < 0.0001$, N=10/genotype. **(c-e)** Area of E17.5 junctional zone,

labyrinth and decidua. **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, N=10/genotype. **(f)**

Representative images of E17.5 junctional zone stained with H&E. Blue arrowheads, glycogen

cells. Scale bars= 500μm. **(g)** Quantification of total number of glycogen cells in E17.5 placenta

cross section. ****$P < 0.0001$, N=10/genotype. **(h)** Gestational length for females producing

litters from *Eed-wt, Eed-het* and *Eed-hom* oocytes. **$P < 0.01$, ***$P < 0.001$, N=6 litters from

Eed-wt oocytes, N=13 litters from *Eed-het* oocytes and N=19 litters from *Eed-hom* oocytes.

(b-e, g-h) statistically analysed using one-way ANOVA plus Tukey's multiple comparisons.

A PCA: *Eed* Placenta vs Controls

PC2 (23%)

PC1 (31%)

Genotype
- *WT-wt*
- *HET-het*
- *HET-hom*

B *HET-het* vs *HET-hom* Placenta DEGs

$-\log_{10}p$

$\log_2$FC

C *HET-het* vs *HET-hom* GO enrichment analysis for the top 10 Biological Processes for *Eed* palcenta DEGs

GO:0048731~system development
GO:0007275~multicellular organism development
GO:0009653~anatomical structure morphogenesis
GO:0009888~tissue development
GO:0048869~cellular develomental process
GO:0048522~positive regulation of cellular process
GO:0051240~positive regulation of multicellular organismal process
GO:0009966~regulation of signal transduction
GO:0010646~regulation of cell communication
GO:2000026~regulation of multicellular organismal development

-log10(*p-value*)

D *Eed* Junctional Zone DEGs

1240   166   377

Junctional Zone Genes   *Eed* Identified Placenta DEGs

E *Eed* Labyrinth DEGs

258   57   486

Labyrinth Genes   *Eed* Identified Placenta DEGs

F *Eed* Decidua DEGs

150   104   439

Decidua Genes   *Eed* Identified Placenta DEGs

G *Eed* Junctional Zone DEGs

23 DEGs
143 DEGs

- Down-regulated
- Up-regulated

H *Eed* Labyrinth DEGs

8 DEGs
49 DEGs

I *Eed* Decidua DEGs

3
101 DEGs

Figure 5

Figure 5. Loss of EED-dependent oocyte programming altered transcription in female *HET-*
***hom* placenta. (a)** Principal Component Analysis (PCA) for female *HET-hom*, *HET-het* and *WT-*
wt placenta. N=4-5/genotype. **(b)** Differential gene expression analysis of isogenic *HET-het* vs
HET-hom placenta represented by a volcano plot showing logFC against statistical
significance. Genes with FDR-adjusted FDR<0.05 are coloured in red. Deletion of *Eed* in the
oocyte resulted in 2083 DEGs in *HET-hom* placenta (*Eed* placental DEGs). **(c)** GO enrichment
analysis of *HET-het* vs *HET-hom* DEGs representing the top 10 significantly different biological
processes impacted **(d-f)** Venn diagrams showing comparisons of: **(d)** identified junctional
zone genes vs *Eed* identified placenta DEGs, **(e)** identified labyrinth genes vs *Eed* identified
placenta DEGs, **(f)** identified decidua genes vs *Eed* identified placenta DEGs. **(g-i)** pie graph
depicting the distribution of up-regulated (pink) and down-regulated (grey) *Eed* DEGs
identified to be expressed in **(g)** junctional zone, **(h)** labyrinth and **(i)** decidual layer of mouse
placenta.
902

A *Eed* E17.5 Placenta DEGs

Mouse Imprinted Genes

H3K27me3 Imprinted Genes

Syt13
Gab1
Hunk
Sfmbt2
Smoc1
Flt1
Bbx
Slc38a4
Slc38a1
Cd44
Myoz2
Tshz2
Rftn1
Adamts2
Gas7
Runx1

B H3K27me3 imprinted genes in E17.5 placenta

Relative expression levels

HET-het
HET-hom

Syt13 Gab1 Hunk Sfmbt2 Smoc1 Flt1 Bbx Slc38a4 Slc38a1

Pat
Mat
Bi

C **Placenta DEGs experssion change by chromosome**

DEGs/Mbp

Chromosome

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 X

D **Expression of Plac1 in E17.5 Placenta (IHC)**

Offspring: WT   HET   HET
Oocyte: wt   het   hom

Figure 6

 Figure 6. Loss of EED in the oocyte results in the de-repression of imprinted and X-linked

genes. (a) Venn diagram showing comparisons of *Eed* placenta DEGs vs known mouse

imprinted genes (37, 38)(bottom left) and H3K27me3 imprinted genes (22) (bottom right). **(b)**

Relative expression levels of H3K7me3 imprinted genes that were upregulated in female

E17.5 *HET-hom* placenta compared to female *HET-het* control (FDR<0.05). **(c)** *Eed* placental

DEG distribution across the genome, represented as DEGs/Mbp for all autosomes and the X-

chromosome. **(d)** Representative images of PLAC1 expression in E17.5 female placenta,

N=4/genotype. Scale bars= 500µm (top), scale bars= 100µm (bottom).

Figure 7

Figure 7. Summary of the placental and offspring growth response due to EED loss in the

oocyte. During oocyte growth all three subunits of PRC2 are present at the primary to

secondary stages, which is important for the silencing of developmental genes and

H3K27me3-dependent imprinting (Jarred et al., 2022, submitted). Deletion of *Eed* in oocyte

results in loss of maternal H3K27me3 and PRC2, early growth restriction, followed by

placental hyperplasia and late gestation fetal catch-up growth, outcomes consistent with loss

of H3K27me3-dependent imprinting observed in SCNT offspring (25-27). Placentas generated

from oocytes lacking EED have expanded glycogen enriched cells in the junctional zone and

significant gene dysregulation in the placenta. Placental hyperplasia immediately precedes

rapid fetal catch-up growth, explaining why these offspring are overgrown immediately after

birth (7). Figure created with BioRender.com.

<u>***Supplementary Data and Information***</u>

Loss of EED in the oocyte causes initial fetal growth restriction followed by placental

hyperplasia and offspring overgrowth.

Short title: *Epigenetic regulation of fetal growth restriction, placental expansion and*
offspring overgrowth
933
934

935 **Authors:** <u>Ruby Oberin[1#], Sigrid Petautschnig[1#]</u>, Tesha Tsai[1^], Zhipeng Qu[2], Ellen G. Jarred[1],
936 Heidi Bildsoe[1], Thi T. Truong[3], Dilini Fernando[1], Maarten van den Buuse[4], David K. Gardner[3],
937 Natalie A. Sims[5], David L. Adelson[2] and Patrick S. Western[1]*

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942 **Supplementary Tables** can be found in associated Excel File: Oberin and Petautschnig *etal.*
943 Supplementary Tables
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946 **Supplementary Figures** are found below

A *Eed* Offspring: Proportion of embryos per female surviving to-

Expanded Blastocyst

Hatched Blastocyst

Proportion of embryos (%)

ns

Offspring: WT / HET
Oocyte: wt / hom

B Differential Stained Blastocyst

C Cell number (TE)

ns

No. of cells

Offspring: WT / HET
Oocyte: wt / hom

D Cell number (ICM)

****

No. of cells

WT / HET
wt / hom

Supp. Figure 1

 Supplementary Figure Legends

Supplementary Figure 1. Maternal *Eed* deletion impacted pre-implantation development.

(a) Proportion of embryos from *Eed-wt* or *Eed-hom* oocytes surviving to expanded blastocyst

(left) and hatched blastocyst (right). Data represent the proportion of surviving embryos from

each female, N = 6 females. **(b)** Example of a differentially stained blastocyst that has been

treated with propidium iodide followed by bisbenzimide to identify purple cells representing

inner cell mass (ICM) and pink cells representing trophectoderm (TE). **(c)** Number of TE cells

per embryo. ns = no significant difference, P > 0.05, N = 17 embryos from *Eed-wt* oocytes, 28

embryos from *Eed-hom* oocytes. **(d)** Number of ICM cells per embryo. ****P < 0.0001, N = 17

embryos from *Eed-wt* oocytes, 28 embryos from *Eed-hom* oocytes. For **(a, c-d)** a two-tailed

student's t test was used, and error bars represent mean ± SD.

A E9.5 Embryo + Extraembryonic tissue weight

Weight (g)

Genotype of mother

B E14.5 Forelimb

Offspring: *WT*   *HET*   *HET*
Oocyte: *wt*   *het*   *hom*

C *HET* offspring weight vs age

Weight (g)

Offspring age

— HET-*het*
— HET-*hom*

D No. reabsrobed embryos at E17.5 per litter

No. reabsorbed embryos

Genotype of mother

WT   HET   HOM

Supp. Figure 2

Supplementary Figure 2. Loss of EED-dependent oocyte programming resulted in altered

fetal and neonatal development. (a) Embryo and extraembryonic tissue weight at E9.5.

****P<0.0001, ***P<0.0005, N=17-40/genotype. **(b)** Representative wholemount images of

E14.5 forelimbs revealing reduced inter-digital tissue regression in *HET-hom* offspring. **(c)**

Isogenic heterozygous offspring average weight over time. ***P<0.0005, ****P<0.0001

unpaired t-test, N=28-56/genotype. **(d)** Number of reabsorbed embryos per litter at E17.5.

****P<0.0001 N=10-15 litters/genotype. **(a, c, e,)** statistically analysed using one-way ANOVA

plus Tukey's multiple comparisons.

A E14.5 Placental Area

B E14.5 Junctional Zone Raw Area

C E14.5 Labryinth Raw Area

D E14.5 Decidua Raw Area

E Placental morphology (PAS stain)

E14.5

Offspring: WT / WT / HET / HET
Oocyte: wt / het / het / hom

F E17.5 Junctional Zone Raw Area

G E17.5 Labryinth Raw Area

H E17.5 Decidua Raw Area

Supp. Figure 3

Supplementary Figure 3. Complete deletion of *Eed* in the oocyte resulted in altered

placental size. (a) E14.5 placenta cross-sectional area. *$P<0.05$, N=10/genotype. **(b-d)** Raw

area of E14.5 junctional zone, labyrinth and decidua. *$P<0.05$, N=10/genotype. **(e)** Placental

cross-sections at E14.5 stained with PAS. Scale bars= 800µm. **(f-h)** Raw area of E17.5

junctional zone, labyrinth and decidua. ****$P<0.0001$, ***$P<0.0005$, *$P<0.05$,

N=10/genotype. Statistically analysed using one-way ANOVA plus Tukey's multiple

comparisons.

A PCA: *Eed* Placenta vs Controls

PC2 (16%)

PC1 (24%)

Genotype
- *Male WT-wt*
- *Male HET-het*
- *Male HET-hom*
- *Female WT-wt*
- *Female HET-het*
- *Female HET-hom*

B *Eed* E17.5 Male Placental DEGs

$-\log_{10}P$

$\log_2 FC$

CAAA01077340.1

Alfm3 Lpin1
Gm4755 Syk
Pri5a1 Gm28437
Scn4b

Nfkbid

Gabrg3

Supp. Figure 4

Supplementary Figure 4. Loss of EED-dependent oocyte programming altered transcription

in male *HET-hom* placenta. (a) Principal Component Analysis (PCA) for *HET-hom*, *HET-het*

and *WT-wt* placenta. N=3-4/genotype. **(b)** Differential gene expression analysis of isogenic

HET-het vs *HET-hom* placenta represented by a MD plot showing $\log_2$FC against statistical

significance. Gene with FDR-adjusted FDR<0.05 is coloured in red. Deletion of *Eed* in the

oocyte resulted in one DEG in male *HET-hom* placenta.

A   **X-linked and imprinted genes in E17.5 placenta**

Supp. Figure 5

983   **Supplementary Figure 5. Loss of EED in the oocyte results in the de-repression of X-linked**

984   **and imprinted genes that are important for placental development. (a)** Relative expression

985   levels of X-linked and imprinted genes in female E17.5 *HET-hom* placenta compared to female

986   *HET-het* control (FDR<0.05).

A   E14.5

B   E19.5

Supp. Figure 6

988   **Supplementary Figure 6. *HET-hom* offspring demonstrate similar growth profiles to SCNT**

989   **mice.** Comparison of body weights of SCNT mice from Xie, Zhang (27) and offspring from the

990   *Eed-ZP3*-Cre mouse model at **(a)** E14.5 and **(b)** E19.5. ****$P<0.0001$, **$P<0.005$ and *$P<0.05$.

991   Statistically analysed using one-way ANOVA plus Tukey's multiple comparisons.