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Loss of POLR1D results in embryonic lethality prior to blastocyst formation in mice

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Abstract

In eukaryotic cells, RNA Polymerase (Pol) I and Pol III are dedicated to the synthesis of ribosomal RNA precursors and a variety of small RNAs, respectively. Although RNA Pol I and Pol III complexes are crucial for regulation of cell growth and cell cycle in all cell types, many of the components of the Pol I and Pol III complexes have not been functionally characterized in mammals. Here we provide the first *in vivo* functional characterization of POLR1D, a subunit shared by RNA Pol I and Pol III, during early mammalian embryo development. Our results show that *Polr1d* mutant embryos cannot be recovered at E7.5 early post-gastrulation stage, suggesting failed implantation. Although *Polr1d* mutants can be recovered at E3.5, they exhibit delayed/stalled development with morula morphology rather than differentiation into blastocysts. Even with extended time in culture, mutant embryos fail to form blastocysts and eventually die. Analysis of E3.0 embryos revealed severe DNA damage in *Polr1d* mutants. Additionally, lineage assessment reveals that trophectoderm specification is compromised in the absence of *Polr1d*. In summary, these findings demonstrate the essential role of POLR1D during early mammalian embryogenesis and highlight cell-lethal phenotype without *Polr1d* function.

Keywords

Blastocyst embryo; Trophectoderm; Cell lineage; RNA Polymerase; DNA damage

Introduction

During mammalian preimplantation embryo development, the fertilized oocyte undergoes three major transcriptional and morphogenetic events to form a competent blastocyst that is capable of uterine implantation (Arny et al. 1987). The first event, maternal-to-zygotic

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Authors' contributions

X. Miao performed the majority of the experiments and analyzed the data; T. Sun and M. Golan contributed to genotyping; J. Mager performed E7.5 embryo dissection, initial outgrowth experiments and helped with manuscript preparation; W. Cui designed the experiments, analyzed the data and wrote the manuscript. All authors reviewed the manuscript.

Conflict of Interest

The authors have declared that no conflict of interest exists.

transition (MZT), initiates the degradation of maternal mRNAs and proteins with replacement from zygotic transcripts (Latham et al. 1991; Schall et al. 2019). Upon reaching 8-cell stage in the mouse, embryo initiates the second major event, compaction and polarization (Houliston and Maro 1989; Sutherland and Calarco-Gillam 1983). The third critical event is blastomere allocation and the first cell-fate determination into the inner cell mass (ICM) and trophectoderm (TE). During this process, the apolar cells located inside of the morula give rise to the ICM, from which the embryo proper and parietal yolk sac are derived (Frum and Ralston 2015); while the outer polar cells differentiate exclusively into the TE, from which extra-embryonic tissues are derived (Fleming 1987; Hogan and Tilly 1978).

Lineage specification during early mammalian embryogenesis is a highly regulated process that relies on the differential expression of various genes within distinct cell populations (Cui and Mager 2018; Paul and Knott 2014). Specific localization of transcription factors (TFs) and differential gene expression profiles have been illustrated in blastocyst embryos of different mammalian species (Iqbal et al. 2014; Negron-Perez et al. 2017). For example, to establish and maintain the cell fate in mouse embryos, the TF OCT4 (also known as POU5F1) is enriched in ICM and functions to promote pluripotency and inhibit differentiation, while the TF CDX2 becomes highly expressed in outer cells of the morula to drive TE differentiation (Niwa et al. 2005). During morula-to-blastocyst transition, TE performs as a fluid transporting epithelium that is responsible for forming the blastocyst cavity, which is also essential for continued blastocyst development (Biggers et al. 1988; Marikawa and Alarcon 2012). Furthermore, aquaporins contribute to water influx through the TE, facilitating the blastocoel expansion and future hatching of the embryo from the zona pellucida (Barcroft et al. 2003). Through advances in genome editing and loss-of-function studies, increasing numbers of genes have been found to be required for TE lineage specification. For example, members of Hippo signaling pathway (Strumpf et al. 2005; Yagi et al. 2007), Notch (Rayon et al. 2014) and ROCK signaling (Kono et al. 2014; Negron-Perez et al. 2018), as well as epigenetic regulators (Chen et al. 2016; Chung et al. 2017) and newly discovered factors (Cao et al. 2015; Cui et al. 2016; Midic et al. 2018; Wang et al. 2020), are known to contribute to TE fate acquisition and expression of the key TF CDX2.

During mammalian preimplantation development, RNA Polymerase (Pol) I, II, and III are involved in transcription of more than 11,000 genes (Siracusa 1973; Xue et al. 2013). In eukaryotes, RNA Pol I is dedicated to the synthesis of ribosomal RNA (rRNA) precursors, Pol II normally produces messenger RNAs (mRNAs) which are translated into proteins, and Pol III synthesizes the transfer RNAs (tRNAs), 5S rRNA and a variety of other small RNAs (Goodfellow and Zomerdijk 2013). It is generally believed that RNA Polymerases are required in all tissues and cell lineages. However, the functional roles of individual subunits comprising each RNA Polymerase remain largely undocumented (Aspesi and Ellis 2019; Yelick and Trainor 2015). Notably, previous reports and our recent study on *Med20* demonstrated that while the Mediator complex is crucial for all Pol II transcription in eukaryotic organisms, the phenotypes of individual Mediator subunit mutants are each distinct (Cui et al. 2019; Yin and Wang 2014). As an extension, we wonder if individual subunits of RNA Pol I or III may also target distinct genes and cell populations.

RNA polymerase I polypeptide D (POLR1D) is an evolutionarily conserved protein that was initially identified in yeast as a subunit of RNA Pol I (Yao et al. 1996). POLR1D has also been shown to be a subunit of RNA Pol III and *POLR1D* heterozygous mutations in humans contribute to Treacher Collins syndrome (TCS) (Dauwerse et al. 2011). A zebrafish model shows that *polr1d* loss-of-function results in deficient ribosome biogenesis and a deficiency of migrating neural crest cells, the primary progenitors of the craniofacial skeleton. These *polr1d* mutant zebrafish embryos die between 9-10 days post fertilization (Noack Watt et al. 2016). POLR1D has also been shown to promote cancer progression both *in vitro* and *in vivo*. Interestingly, all these reports have been centered on colorectal cancer only but no other types (Camps et al. 2013; Sheffer et al. 2009; Wang et al. 2019; Zhou et al. 2020).

In the present study, we use a novel knockout (KO) allele to explore the role of POLR1D during murine embryogenesis *in vivo*. Our data show that POLR1D is essential during early preimplantation development, where loss of POLR1D results in DNA damage, impaired TE specification, and failure to form a blastocyst.

Materials and Methods

All chemicals and media were purchased from Millipore-Sigma (Burlington, MA, USA) unless otherwise indicated.

Generation of *Polr1d* mutants

All procedures and methods were carried out in accordance with the approved guidelines and regulations. All animal experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Massachusetts, Amherst (2017-0071). *Polr1d* KO allele (C57BL/6NJ-$^{Polr1d\ em1(IMPC)}$/Mmjax, MMRRC Stock No: 42303-JAX) was generated on C57BL/6NJ background by the Knockout Mouse Phenotyping Program at the Jackson Laboratory with CRISPR technology, resulting in a 938 bp deletion beginning at Chromosome 5 positive strand position 147,078,255 bp, CATCTTGCAGCAGGGCCTAC, and ending after AACAGATAATTGGGCCAAGG at 147,079,192 bp (GRCm38/mm10). With the entirety of exon 2 (ENSMUSE00000346921) deleted from the *Polr1d* gene (ENSMUST00000050970.3), this KO allele only generates the first 8 amino acids (Fig. 1A). Correctly targeted pups were identified by PCR at the Jackson Laboratory and further backcrossed with C57BL/6N to develop the colony. To expand the colony in our animal facility for the present study, sequence confirmed heterozygous *Polr1d−/Polr1d+* (hereafter referred to as Het) mice from the Jackson Laboratory were backcrossed with C57BL/6N wildtype *Polr1d+/Polr1d+* (hereafter referred to as WT) mice, and subsequent Het mice were intercrossed to generate *Polr1d-/Polr1d-* mutants (hereafter referred to as Mut). Genotyping primers include: Forward for WT allele: 5′-TAGATGAACCCCAAGGATGG; Forward for Mut allele: 5′-CTGCCACCCTGATAACTTGTG; common Reverse for WT allele and Mut allele: 5′-AAAAATCTATGATCAAAACCCCTA.

Embryo recovery, outgrowth culture and genotyping

Polr1d heterozygous females (8- to 14-weeks old) were caged overnight with *Polr1d* heterozygous males and examined for the presence of copulatory plugs each morning. The

presence of a plug was defined as embryonic day 0.5 (E0.5). Embryos were collected from heterozygous females by dissection or flushing to collect E7.5 or E3.5 embryos, respectively. For E7.5 embryos, embryos were imaged after dissection, then collected into individual tubes for lysis and PCR genotyping. For E3.5, embryos were imaged individually in droplets and then cultured for 3 days in DMEM (Lonza, Allendale, NJ, USA) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Flowery Branch, GA, USA) and 1X GlutaMAX (Thermo Fisher Scientific, Waltham, MA, USA). After 3 days of culture, outgrowths were individually imaged again before lysis and genotyping. Outgrowth analysis was performed as described previously (Cui et al. 2019). Briefly, an outgrowth that displayed a distinctive ICM colony surrounded by trophoblast monolayer is considered a normal successful outgrowth, while outgrowths that fail to hatch, lack ICM colony or lack trophoblast monolayer are considered as failed outgrowths.

Immunofluorescence

Immunofluorescence staining (IF) was carried out in accordance with the methods of our previous studies (Miao et al. 2020). E3.0 morula embryos were harvested and then fixed directly in 4% paraformaldehyde. After brief wash in PBS, embryos were permeabilized with 0.5% Triton X-100 for 20 min. Embryos were then blocked in blocking solution (PBS with 10% FBS and 0.1% Triton) for 1 h at room temperature, and then incubated with primary antibodies overnight at 4 °C. All primary antibodies were diluted 1:200 using the blocking solution, including: goat anti-OCT4 (abcam, ab27985); mouse anti-CDX2 (BioGenex, MU392A-UC); rabbit anti-TRP53 (Cell Signaling Technology, #9284). After 3 washes, embryos were incubated with suitable secondary antibodies (Alexa Fluor, Thermo Fisher Scientific, Waltham, MA, USA) for 1 h at room temperature in dark. After 2 washes, DNA was stained with 4,6-diamidino-2-phenylindole (DAPI) before transferring embryos into single wells of chambered slides (Corning Co., Corning, NY, USA) for imaging under a Nikon A1 Spectral Detector Confocal with FLIM Module. Z-stacks (20X objective, 8 μm sections) were collected and maximum projection applied. Embryos were handled individually such that each one was imaged and then recovered for PCR genotyping.

TUNEL labeling

TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) staining was carried out using the *In Situ* Cell Death Detection Kit (11684795910, Roche) according to the manual. Embryos were washed three times and labeled with TUNEL reaction mixture in the dark at 37°C for 30 min. Then DNA was stained with DAPI for 10 min before embryos were individually transferred into single wells of chambered slides for imaging under confocal as described in the IF method section.

Detection of reactive oxygen species (ROS)

CellROX® Green Reagent (C10444, Thermo Fisher Scientific) was used to detect ROS level according to the product manual. Embryos were incubated in KSOM supplemented with 5 μM CellROX® Green Reagent at 37°C for 30 min. After this, embryos were washed 3 times with PBS and then fixed in 4% paraformaldehyde for 20 min. Fixed embryos were then washed and stained with DAPI, and then individually transferred into single wells of chambered slides for imaging as described in the IF method section.

RNA extraction and Reverse Transcription PCR (RT-PCR)

Total RNA extraction was carried out with a Roche High Pure RNA Isolation Kit (#11828665001). cDNA was synthesized using iScript cDNA synthesis kit (#170-8891; Bio-Rad Laboratories, Hercules, CA, USA). Intron-spanning primers used for RT-PCR: (*Actb*: 5'-GGCCCAGAGCAAGAGAGGTATCC and 5'-ACGCACGATTTCCCTCTCAGC; *Polr1d*: 5′-ACGATCAGGAGCTGGAGAGA and 5′-TGCTGGCAGACATTCAAGAG).

Results

Polr1d mutants cannot be recovered *in vivo* at E7.5

As detailed in Methods, the entirety of exon 2 (ENSMUSE00000346921) was deleted from the *Polr1d* gene (ENSMUST00000050970.3) by the Knockout Mouse Phenotyping Program at the Jackson Laboratory with CRISPR technology, and this KO allele only generates the first 8 amino acids (Fig. 1A). The initial phenotyping analysis performed for the International Mouse Phenotyping Consortium (IMPC) (Dickinson et al. 2016) indicated no homozygous *Polr1d* mutants (*Polr1d−/Polr1d−*) were born, nor found at E15.5 or E12.5 (http://www.mousephenotype.org). Therefore, we first dissected embryos at E7.5. Thirty-seven embryos were recovered from five heterozygous intercrosses. Genotyping revealed 28 Het (*Polr1d−/Polr1d+*) and 9 WT (*Polr1d+/Polr1d+*) embryos, with no obvious difference between these 2 genotypes (Fig. 1B). No *Polr1d* homozygous mutant embryos were found, nor was there any empty decidua (n=0), suggesting that *Polr1d* mutants fail to implant.

E3.5 *Polr1d* mutants exhibit retarded development

To gain more insight into the mechanism underlying the embryonic lethality *in vivo*, we collected E3.5 embryos for morphological assessment and 3-day-outgrowth (OG) assays. Among 30 embryos, 6 *Polr1d* mutants were recovered along with 9 WT and 15 Het embryos. Compared with WT and Het embryos that all reached blastocyst stage with visible blastocoel cavities, none of the Mut embryos exhibited signs of cavitation. Instead, all Mut embryos displayed a compacted morula morphology (Fig. 2A). Outgrowth assays revealed that while successful outgrowth rates were high for WT (6/9) and Het (11/15), none of the mutants (0/6) formed an outgrowth. Moreover, none of mutants formed a blastocyst during the 3-day extended culture. By the end of culture, all mutants had a necrotic/lysed morphology still trapped inside the zona pellucida (Fig. 2A, B), indicating the essential role of POLR1D in blastomere survival and blastocyst formation. These results are consistent with a complete absence of mutant embryos at E7.5 *in vivo*. Based on the phenotype and timing of lethality, we evaluated *Polr1d* expression during preimplantation stages. Due to the lack of available antibodies suitable for immunofluorescence, we performed RT-PCR and revealed that *Polr1d* mRNA is present at all stages examined (Fig. 2C).

Polr1d mutants fail to specify TE

To investigate the mechanisms underlying blastulation failure *in vivo* and outgrowth lethality *in vitro*, we collected embryos at E3.0 to examine markers of the first cell lineage specification (OCT4 for ICM and CDX2 for TE) and apoptosis (active TRP53 with phosphorylation at Ser15). None of the 18 morulae from heterozygous intercrosses (3 WT,

12 Het, 3 Mut) showed obvious TRP53-positive cells. Although OCT4 expression was comparable in *Polr1d* mutants, CDX2 expression was strikingly decreased when compared with WT and Het littermates (Fig. 3), suggesting the specification of TE, which is responsible for fluid influx and blastocoel formation, is impaired due to loss of POLR1D.

Polr1d mutants display many DNA breaks

Given that TUNEL staining (marker of DNA damage and cell death) is significantly increased in *polr1d*$^{-/-}$ zebrafish embryos (Noack Watt et al. 2016), we also performed fluorescent whole-mount TUNEL assays to evaluate DNA breaks in E3.0 morula embryos. Among 28 embryos genotyped (5 WT, 18 Het, 5 Mut), all *Polr1d* mutant embryos exhibited many TUNEL positive blastomeres, indicating many DNA breaks (almost none in littermates, Fig. 4A). We next assessed the levels of reactive oxygen species (ROS), which has been used as marker for oxidative homeostasis (Zhang et al. 2018). Among 26 embryos examined (5 WT, 18 Het, 3 Mut), all genotypes exhibited similarly low levels of ROS (Fig. 4B), suggesting that POLR1D does not significantly regulate or alter oxidative homeostasis during early mammalian embryogenesis.

Discussion

Ribosomes are macromolecular machines, universally responsible for the quality and quantity of proteins in all cells. Therefore, ribosome biogenesis is a global process required for growth and proliferation of all cells (Noack Watt et al. 2016; Yelick and Trainor 2015). In eukaryotes, ribosome biogenesis involves all three RNA polymerases. RNA Pol I is required for the synthesis of rRNA precursors that give rise to 18S, 5.8S, and 28S rRNAs; RNA Pol II produces mRNAs for all ribosomal proteins; and RNA Pol III produces 5S rRNA (Aspesi and Ellis 2019). As a subunit shared by RNA Pol I and Pol III, POLR1D has been documented as a key component during rRNA production and ribosome biogenesis. Particularly, *POLR1D* heterozygous mutation has implications in the ribosomopathy, Treacher Collins syndrome (TCS), a congenital disorder characterized by anomalies of the facial bones, palate, eyes and ears (Dauwerse et al. 2011). In the present study, we demonstrate the first *in vivo* functional characterization of POLR1D during early mammalian embryo development. The phenotype of delayed development at E3.5 is consistent with its key function in rRNA production and ribosome biogenesis illustrated previously in other organisms (Aspesi and Ellis 2019; Dauwerse et al. 2011; Wang et al. 2019).

In zebrafish, *polr1d* loss-of-function results in a deficiency of migrating neural crest cells, the primary progenitors of the craniofacial skeleton. In addition, strikingly increased DNA damage and cell death was shown in the cranial region of *polr1d*$^{-/-}$ zebrafish, contributing to the embryonic lethality between 9-10 days post fertilization (Noack Watt et al. 2016). In our study, we also observed greatly increased DNA breaks and cell death in *Polr1d* mutant (*Polr1d-/Polr1d-*) mouse embryos (based on TUNEL staining), but the cell death seems not TRP53-dependent (based on IF of active TRP53 with phosphorylation at Ser15), which is different from *polr1d*$^{-/-}$ zebrafish. Also, the stage of lethality in mice (before blastulation and implantation in this study) is much earlier than that in zebrafish (after organogenesis)

(Noack Watt et al. 2016). We speculate this could be due to the different amount of maternally loaded protein or different lifespan of the protein after fertilization. Another plausible explanation is that POLR1D protein may exert differential importance or necessity among different species. Indeed, heterozygous mutation in *POLR1D* causes a deficiency in migrating neural crest cells in human, but heterozygous mice appear normal and are viable and fertile. Interestingly, the TCS phenotype can be recapitulated in a zebrafish model, but does require homozygous mutation (Noack Watt et al. 2016).

Historically, ribosome biogenesis has been considered as a tightly regulated global process, which performs a constitutive but not regulatory role in mRNA translation. However, in the present study, loss of POLR1D severely impairs CDX2 expression and TE specification, but not on OCT4 expression and ICM lineage development. This lineage-specific necessity of one particular Pol I/III subunit is interesting, and several plausible scenarios could exist underlying this phenomenon. One scenario is that ribosome biogenesis is spatiotemporally dynamic and TE specification requires higher threshold level of ribosomal activity to translate relevant key TFs such as CDX2. This hypothesis has also been postulated in the pathogenesis of TCS, given that only neuroepithelial cells and neural crest cells are affected during the embryo development (Dixon et al. 2006; Noack Watt et al. 2016). Another scenario is that lineage-specific ribosomes themselves may be specialized, which consist of diverse rRNA and ribosomal protein combinations, biasing the translation onto a select subset of mRNAs. For example, mutations of the Ribosomal Protein L38 (*Rpl38*) gene in mice does not alter the global protein synthesis, however, *Rpl38* KO interferes the translation of Homeobox mRNAs (Kondrashov et al. 2011). In fact, structure-function analysis of RNA Pol I and III also supports the hypothesis that subunits in the core of RNA Pol I and III may function as part of the basal machinery, while those subunits occupying the periphery (e.g. POLR1D) may provide tissue or activity specificity (Werner et al. 2009; Yelick and Trainor 2015). Collectively, both our present study and previous findings suggest that ribosome biogenesis may serve as a new layer of specificity in the control of gene expression and lineage specification.

In summary, by using the novel KO allele, we first demonstrate that POLR1D is essential for early murine embryogenesis *in vivo* - loss of POLR1D results in severe DNA damage, delayed cellular proliferation, and impaired TE specification, each likely contributing to the embryonic lethality and blastulation failure in mice.

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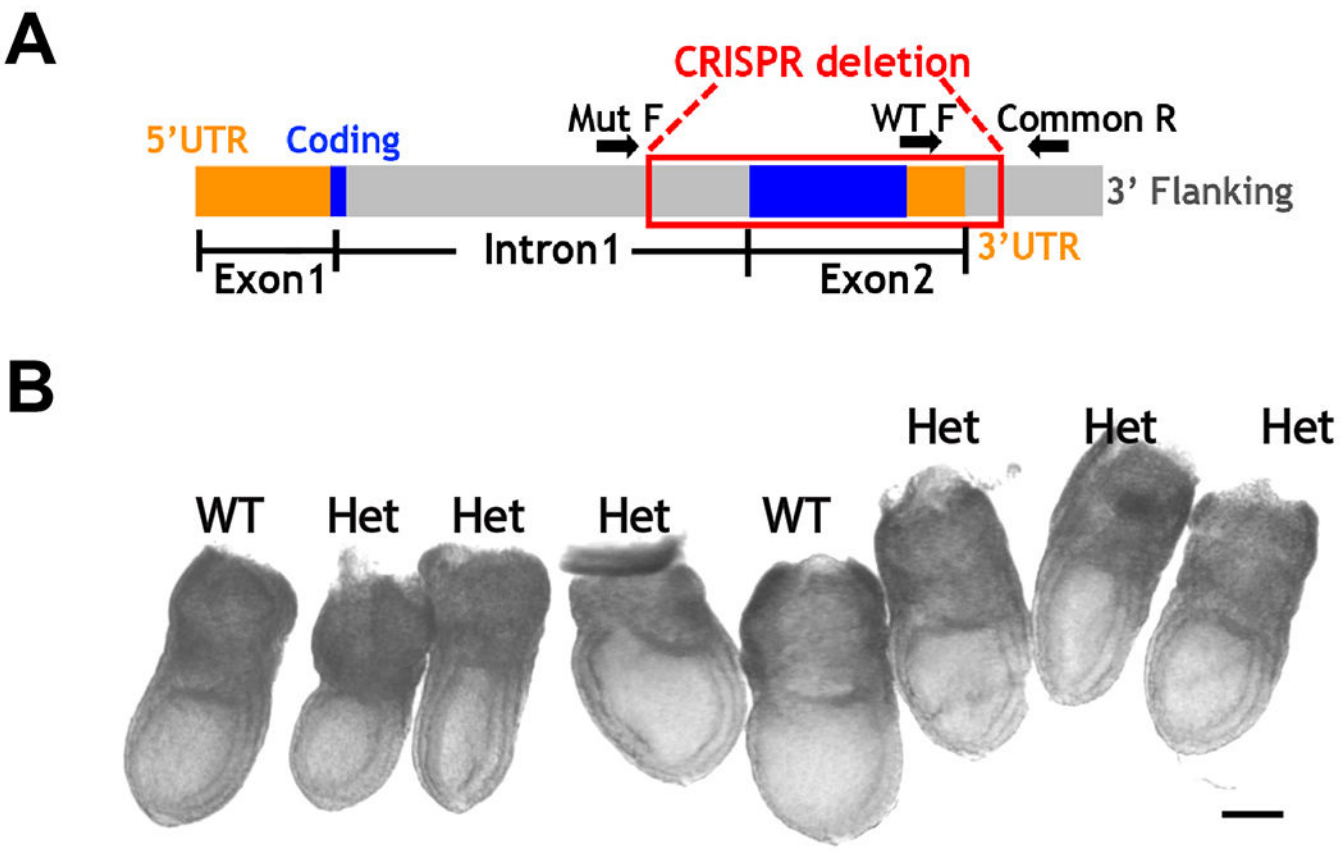

Figure 1.

(A) Schematic of *Polr1d* knock-out allele generation and genotyping primers for WT allele and Mut allele. F, forward; R, reverse. (B) Representative genotyped embryos at E7.5. Scale bar, 100 μm.

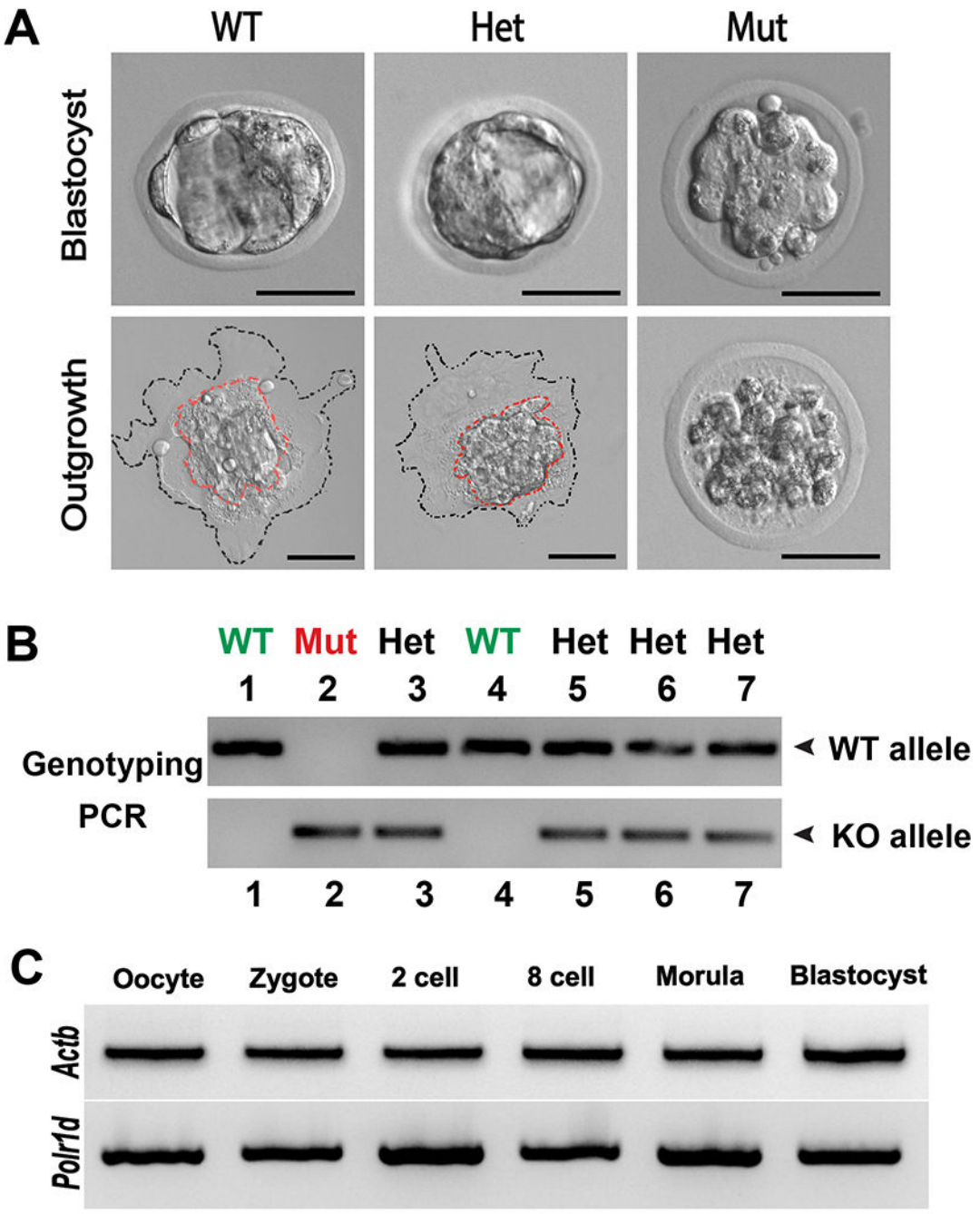

Figure 2.

(A) E3.5 embryos from heterozygous intercrosses were imaged and subjected to outgrowth assays. While WT and Het embryos all reached blastocyst stage at E3.5, all Mut embryos displayed morula morphology. Outgrowths from WT and Het embryos displayed a distinctive ICM colony (red dashed line) surrounded by trophoblast cells (black dashed line). Mutants never formed blastocysts during the outgrowth culture, eventually dying. Scale bars, 50 μm. (B) Representative genotyping PCR of individual outgrowth. (C) RT-PCR showing *Polr1d* expression in WT pre-implantation embryos. *Actb* was used as control.

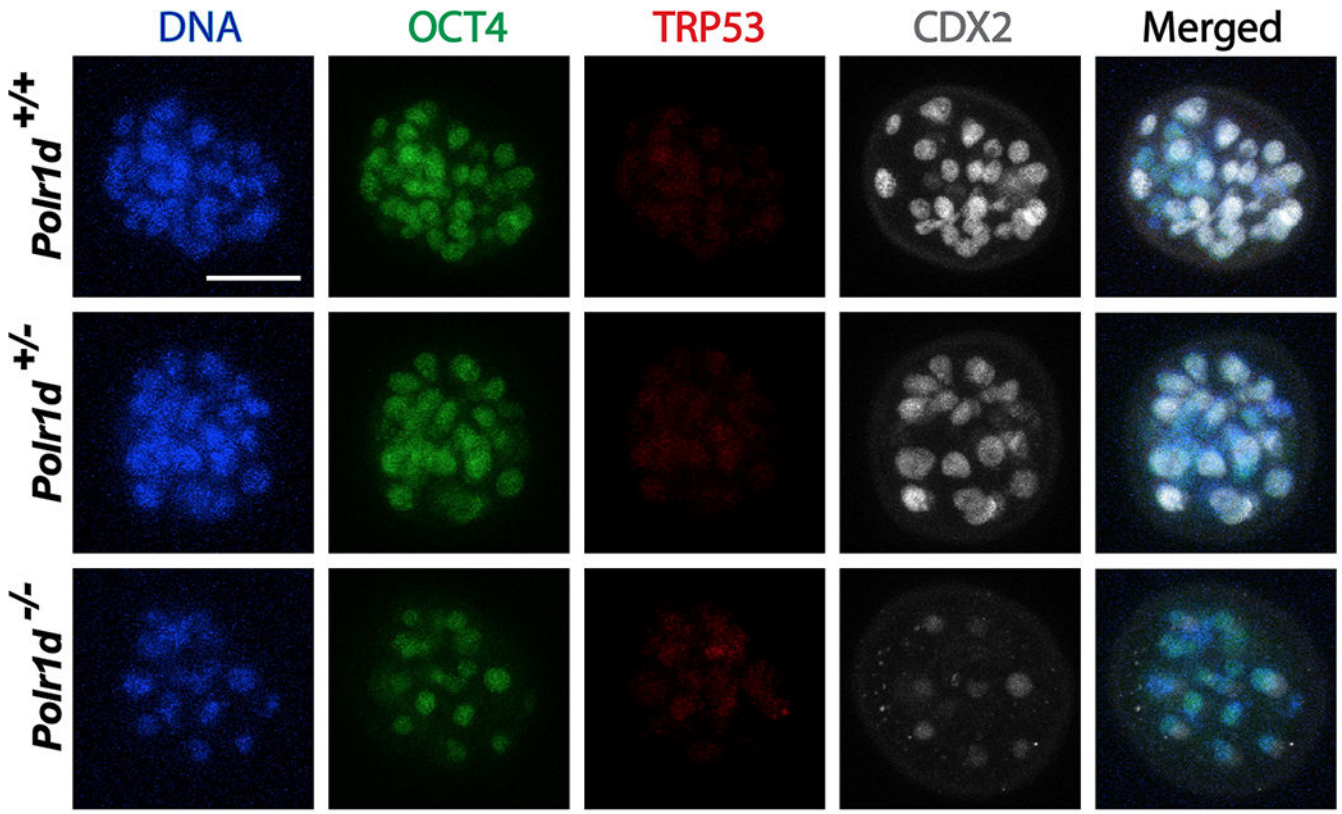

Figure 3.

IF of OCT4 (ICM marker), active TRP53 (apoptosis marker), and CDX2 (TE marker) in E3.0 morulae of different genotypes. None of the embryos showed obvious TRP53-positive cells in all genotypes. OCT4 expression was comparable in *Polr1d* mutants, CDX2 expression was strikingly decreased when compared with WT and Het littermates. Scale bar, 50 μm.

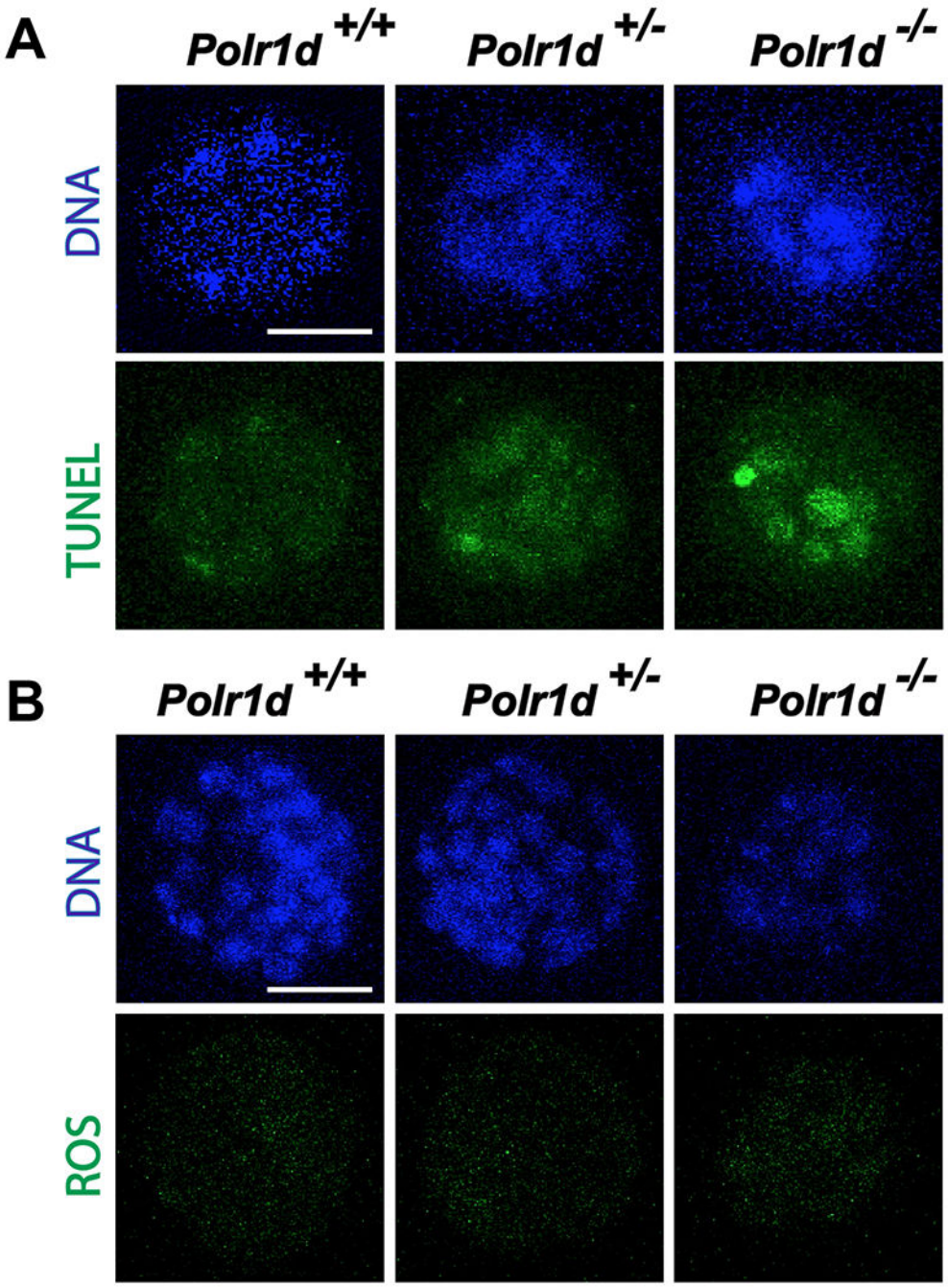

Figure 4.

(A) Fluorescent whole-mount TUNEL assay was performed to evaluate if POLR1D is involved in DNA damage and cell death. (B) IF of ROS (marker for oxidative homeostasis) in embryos of different genotypes. All genotypes displayed similarly low ROS levels, suggesting that embryonic lethality of *Polr1d* mutants is not due to oxidative stress, nor does POLR1D significantly regulate oxidative homeostasis during early embryo development in mice. Scale bars, 50 μm.