frontiers
in Cell and Developmental Biology

REVIEW
published: 29 May 2020
doi: 10.3389/fcell.2020.00416

A Survey of Essential Genome Stability Genes Reveals That Replication Stress Mitigation Is Critical for Peri-Implantation Embryogenesis

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Specialty section:
This article was submitted to Cell Growth and Division, a section of the journal Frontiers in Cell and Developmental Biology

Received: *10 February 2020*
Accepted: *05 May 2020*
Published: *29 May 2020*

Citation:
Kafer GR and Cesare AJ (2020) A Survey of Essential Genome Stability Genes Reveals That Replication Stress Mitigation Is Critical for Peri-Implantation Embryogenesis. Front. Cell Dev. Biol. 8:416. doi: 10.3389/fcell.2020.00416

Murine development demands that pluripotent epiblast stem cells in the peri-implantation embryo increase from approximately 120 to 14,000 cells between embryonic days (E) 4.5 and E7.5. This is possible because epiblast stem cells can complete cell cycles in under 3 h *in vivo*. To ensure conceptus fitness, epiblast cells must undertake this proliferative feat while maintaining genome integrity. How epiblast cells maintain genome health under such an immense proliferation demand remains unclear. To illuminate the contribution of genome stability pathways to early mammalian development we systematically reviewed knockout mouse data from 347 DDR and repair associated genes. Cumulatively, the data indicate that while many DNA repair functions are dispensable in embryogenesis, genes encoding replication stress response and homology directed repair factors are essential specifically during the peri-implantation stage of early development. We discuss the significance of these findings in the context of the unique proliferative demands placed on pluripotent epiblast stem cells.

Keywords: early development, embryology, pluripotency, DNA damage response, DNA repair, DNA replication, replication stress response

INTRODUCTION

Overview

Pluripotent cells in early mammalian embryos proliferate at a phenomenal rate. This is necessary to maintain embryo growth and reach critical developmental milestones within defined temporal windows. Because all somatic tissues are derived from these early pluripotent precursors, it is critical that genome integrity is maintained during early development. Embryonic pluripotent stem cells are thus subjected to unique challenges to maintain their DNA health. To elucidate which genome stability pathways are essential for early development we probed the Mouse Genome Informatics Gene Ontology Project (MGI-GO) database (Bult et al., 2019). Within MGI-GO we identified 347 genes grouped within the ontologies of major DNA repair pathways (MGI-GO designations: DNA damage checkpoint, nucleotide excision repair, mismatch repair, base excision repair, homologous recombination, and non-homologous end joining). Of these genes, we identified 297 with a validated mouse knockout. From these 297 murine models, only 108 gene knockouts were lethal during embryonic development (**Supplementary Table S1**). Within the grouping of 108 embryonic

lethal genes, 10 knockouts were lethal during preimplantation development prior to E4.5 (**Table 1**), and 36 knockouts were lethal during somite stages from E8.5 (**Supplementary Table S1**). Notably, most of the targeted genes that conferred embryonic lethality, 62 genes, did so specifically during the period of rapid cell proliferation occurring with peri-implantation development (E4.5 to E8.5) (**Table 2**). Below we briefly review pre- and peri-implantation murine development before considering the function of essential genome stability factors across the early stages of embryonic development. Finally, we discuss why the unique cells of the peri-implantation embryo appear to specifically require replication stress response factors for cell viability.

Early Murine Development

Embryonic development consists of a series of events occurring in chronological progression. Murine development takes 19 to 20 days depending on mouse strain (**Figure 1**; Murray et al., 2010). Preimplantation development occurs between fertilization (E0) and the initiation of embryo implantation in the uterine wall (around E4.0). Following fertilization, embryonic cells are uniformly totipotent and identical until formation of the morula at E2.5 (Condic, 2014) (see **Box 1** for a detailed explanation of cell potency). Within the morula cells undergo polarization and compaction (Humięcka et al., 2017). By E3.0 the embryo forms a blastocyst structure containing two distinct cell populations; an outer layer of multipotent trophectoderm cells that eventually derive the placental tissues, and an inner group of pluripotent inner cell mass (ICM) cells which primarily serve as precursors for the embryo proper (Morris et al., 2010).

Peri-implantation is the developmental period from implantation to organogenesis (E4.0 to E8.5). Implantation begins at E4.0 when the free-floating embryo contains 64 cells (Behringer et al., 2014). At this point, the embryo loses its glycoprotein zona pellucida shell and the outer trophoblast attaches to the uterine wall. Single cell transcriptomics demonstrate that at E4.0 the ICM has differentiated into two pluripotent cell types: the primitive endoderm (PrE, sometimes referred to as the hypoblast) and the epiblast (Mohammed et al., 2017). Peri-implantation development is associated with exceptionally rapid cell proliferation during "gastrulation," which begins at E5.5 as the embryo elongates and a luminal pro-amniotic cavity opens in the center of the epiblast cell mass (Snow, 1976). At E6.5 the primitive streak emerges from the epiblast, and from the epiblast all three dermal lineages of the conceptus will arise (Tam et al., 1993). These are the ectoderm

BOX 1 | Potency states. Totipotent cells are present in the embryo between fertilization and morula formation. They can give rise to any cell type from any stage of the animal's life, including germ and placental cells. Pluripotent cells exist in the inner cell mass (ICM) and epiblast region of the developing embryo from the blastocyst stage to immediately prior to organogenesis. Pluripotent cells can give rise to any cell of dermal lineage (mesoderm, endoderm or ectoderm) but not placental or germ cells. Multipotent cells exist in newly formed tissues or organs and can develop into a limited number of cell types within their original dermal lineage.

TABLE 1 | Genome stability factors essential for preimplantation development.

Targeted gene	Lethal at	Phenotype details	Gene Function	References
Ercc2 (Xpd)	E1.5	Mutant embryos do not implant. *In vitro* culture indicates embryo failure at the 2-cell stage.	NER*	de Boer et al., 1998
Dtl	E2.5	Embryos develop to the morula stage but do not form blastocysts.	CC, DDR	Liu et al., 2007
Pcna	E2.5	Embryos do not survive to the blastocyst stage.	Rep, BER, NER, MMR	Roa et al., 2008
Rpa1	E2.5	Morula form but do not progress to blastocysts.	Rep, HDR, NER, BER, MMR	Wang et al., 2005
Wee1	E2.5	Morula form but do not progress to blastocysts.	CC, DDR	Tominaga et al., 2006
Nop53 (Pict1)	Before E3.5	Mutant embryos develop morphologically typical morula, but do not form well-structured blastocysts.	CC, DDR*	Sasaki et al., 2011
Cdk1	Before E3.5	Morula form but do not progress to blastocysts.	CC, DDR	Diril et al., 2012
Plk1	E3.5	Mutant embryos develop to the morula stage but do not form viable blastocysts *in vitro*.	CC, DDR	Lu et al., 2008
Xab2	E3.5	Mutant embryos form morula and show some signs of compaction, but do not form blastocysts.	NER*	Yonemasu et al., 2005
Pot1a	After E3.5	Morula are recovered and can be cultured but do not form blastocysts.	Telomere	Hockemeyer et al., 2006

*Key: (E) = Embryonic day; DDR = DNA damage response/damage sensing; CC = cell cycle control; Rep = DNA replication and/or replication stress; HDR = Homologous directed repair; EJ = End joining pathways; BER = Base excision repair; NER = Nucleotide excision repair; MMR = Mismatch repair; Telomere = telomere protein. Gene functions are cross-referenced with the literature and may diverge from the MGI-GO categorization. Factors labeled with * indicate when the gene encodes a protein with additional functions outside genome stability that likely contribute to embryonic lethality.*

TABLE 2 | Genome stability factors essential for peri-implantation development.

Targeted gene	Lethal at	Phenotype details	Gene function	References
Zfp830 (Omcg1)	E3.5	Knockouts blastocysts form but fail to outgrow and do not induce an implantation response *in vivo*.	CC, Rep, DDR*	Artus et al., 2005
Actr2	After E3.5	Lethal after blastocyst stage.	HDR*	Zhang et al., 2007
Cdc7	After E3.5	Blastocysts form and can hatch. Outgrowth assays demonstrate with *in vitro* culture knockout embryos have a small ICM population by E5.5.	CC, Rep, DDR	Kim et al., 2002
Chek1	After E3.5	Blastocysts form *in vivo* but are resorbed following implantation. *In vitro* cultured blastocysts have elevated apoptosis in ICM populations.	Rep, DDR	Takai et al., 2000 Zaugg et al., 2007
Atr	E4.5	Blastocysts form, hatch, and show evidence of initiating implantation *in utero*. ICM outgrowth assays are consistent with wildtype embryos, but ICM populations succumb to apoptosis with continued culture.	CC, Rep, DDR, HDR	de Klein et al., 2000 Brown and Baltimore, 2000 Murga et al., 2009
Cdc25A	E4.5	Blastocysts form but suffer from impaired hatching ability.	CC, Rep	Ray et al., 2007
Ctip (Rbbp8)	E4.5	Embryos form blastocysts but fail to form an egg cylinder. Cells appear to suffer from reduced DNA synthesis.	Rep, HDR	Chen et al., 2005
Recql4	From E4.5	Outcomes are dependent on the genetic manipulation. Deletion of exons 5–8 are lethal during gastrulation, deletion of exon 13 results in lethality just after birth.	Rep, HDR	Hoki et al., 2003
Rint1	From E4.5	Null embryos implant *in vivo* but are severely developmentally delayed and resorbed from E6.5. The ICM and trophoblasts initially outgrow *in vitro*, but proliferation fails after 6 days.	HDR*	Lin et al., 2013
Thoc1	From E4.5	Embryos form a blastocyst, hatch, and attach in *in vitro*, but suffer from reduced proliferation of the ICM after several days in culture. No embryos are recovered *in vivo* before E8.5, but decidua are present, suggesting the embryos die during gastrulation.	CC, HDR, DDR*	Wang et al., 2006
Apex1	After E4.5	Blastocysts form and attach but die soon after.	Rep, BER	Xanthoudakis et al., 1996
Cdc45	After E4.5	Blastocysts form and can hatch. Blastocyst outgrowth assays demonstrate that after several days of culture the ICM mass is smaller or not present.	CC, Rep	Yoshida et al., 2001
Cdk7 (Cdks)	After E4.5	Decidual resorption at peri-implantation stages *in vivo. In vitro* cultured embryos demonstrate increased apoptosis in the blastocyst and severely reduced ICM proliferation.	CC, Rep	Ganuza et al., 2012
Fen1	After E4.5	Blastocysts form and hatch but are compromised during peri-implantation.	Rep, EJ, BER,	Larsen et al., 2003
Gins4 (Sld5)	After E4.5	Embryos form blastocysts that hatch and implant. Development is compromised after implantation as embryos do not form egg cylinders. Embryos attach in outgrowth assays, but after 2 days of culture the ICM becomes compromised.	Rep	Mohri et al., 2013
Hinfp	After E4.5	Knockout blastocysts can form, hatch, and attach, but embryos do not survive past E6.5.	CC, Rep*	Xie et al., 2009
Mdm2	After E4.5	Embryos can implant but are quickly resorbed.	DDR	Jones et al., 1995
Mnat1	After E4.5	Blastocysts form that are indistinguishable from wildtype littermates, but no embryos are found after gastrulation.	CC, Rep*	Rossi et al., 2001

(Continued)

TABLE 2 | Continued

Targeted gene	Lethal at	Phenotype details	Gene function	References
Pold3	After E4.5	Blastocysts form, hatch, and attach *in vitro* but show reduced outgrowth compared to wildtype embryos. *In vivo*, no embryos are recovered at E7.5 consistent with embryo failure during gastrulation.	Rep, NER, TLS	Zhou et al., 2018
Prpf19	After E4.5	Same as above.	CC, Rep, DDR, HDR	Fortschegger et al., 2007
Ptpn11	After E4.5	Same as above.	CC*	Yang et al., 2006
Topbp1	After E4.5	Blastocysts form and hatch but are unable to attach to the tissue culture dish *in vitro*.	CC, Rep, DDR, HDR	Jeon et al., 2011
Yy1	From E5.0	Knockout blastocysts, form, hatch, and outgrow normally *in vitro.* However, *in vivo*, embryos fail to form and egg cylinder and die during gastrulation.	DDR, HDR*	Affar El et al., 2006
Rad51	Before E5.5	Blastocysts appear normal, but post-implantation embryos fail to properly develop an amniotic cavity, display no discernible mesoderm, have a reduced proliferation rate, and elevated apoptosis.	Rep, HDR	Lim and Hasty, 1996 Tsuzuki et al., 1996
Thoc5	Before E5.5	Embryos are not recovered from the uterus from E5.5 suggestive of death during preimplantation or gastrulation.	CC, HDR, DDR*	Mancini et al., 2010
Timeless	From E5.5	Embryos display severe cellular disorganization during gastrulation.	Rep	Gotter et al., 2000
Brca1	E5.5	Growth arrest and impaired proliferation resulting in resorption.	Rep, HDR	Hakem et al., 1996 Ludwig et al., 1997
Mre11A	E5.5	Embryos compromised during gastrulation.	Rep, HDR, DDR, EJ	Xiao and Weaver, 1997 Theunissen et al., 2003 Buis et al., 2008
Nbn (Nbs1)	E5.5	Blastocysts form and hatch, but embryos are smaller after implantation and are resorbed around gastrulation.	Rep, DDR, HDR, EJ	Zhu et al., 2001
Ube2N	E5.5	Embryos are never recovered, and timed mating suggests that embryos die during gastrulation.	HDR	Fukushima et al., 2007
Rad50	Before E6.0	Embryos are abnormal from gastrulation onset and are resorbed.	Rep, DDR, HDR, EJ	Luo et al., 1999
Fh1	E6.0	Embryos do not appear to grow past the early egg cylinder stage.	NHEJ*	Pollard et al., 2007
Blm	E6.5	Reduced embryo size, epiblast population and mesoderm population. The primitive streak forms but there is increased apoptosis throughout the embryo.	HDR, Rep	Chester et al., 1998
Cops5	E6.5	Gastrulating embryos are smaller and cannot develop all germ layers. Embryos display elevated apoptosis.	CC, DDR*	Tomoda et al., 2004
Kdm1A	E6.5	Blastocysts form, hatch, and attach *in vitro*, but are developmentally stunted from E6.5.	DDR, HDR*	Foster et al., 2010
Usp7	E6.5	Embryos die during gastrulation due to reduced proliferation.	Rep, NER	Kon et al., 2010
Xrcc1	E6.5	Epiblast cell numbers are reduced in early gastrulating embryos due to apoptosis and slow proliferation, which impacts lineage specification.	EJ, BER	Tebbs et al., 1999
Pold1	Before E7.5	Knockout embryos form blastocysts at E4.5 but no embryos are retrieved at E7.5.	Rep, BER, NER, MMR	Uchimura et al., 2009
Cul4A	Before E7.5	Embryos form blastocysts *in vitro* that hatch from the zona pellucida with no ICM or trophoblast compromise. No characterization of lethality presented.	CC, Rep, DDR, NER	Li et al., 2002
Dna2	Before E7.5	No embryos retrieved after E7.5. The cause of lethality was not investigated further.	Rep, HDR, BER	Lin et al., 2013
Rif1	From E7.5	Null embryos are lethal from gastrulation in some genetic backgrounds but are viable in different mouse strains. Dimorphism in embryo survivability observed where male knockouts survive but female knockouts do not.	Rep, HDR	Chapman et al., 2013

(Continued)

TABLE 2 | Continued

Targeted gene	Lethal at	Phenotype details	Gene function	References
Bard1	E7.5	Embryos do not develop past egg cylinder stage.	Rep, HDR	McCarthy et al., 2003 Shakya et al., 2008
Brca2	E7.5	Reduced embryo size and persisting Oct4 positive egg cylinder indicates retarded dermal commitment of epiblast cells.	Rep, HDR	Sharan et al., 1997
Hus1	E7.5	Gastrulating embryos appear normal but are smaller than wildtype littermates. Development becomes severely delayed from mid-gastrulation.	Rep, DDR, BER, NER	Weiss et al., 2000
Ino80	E7.5	Knockout embryos implant as deciduas are found at E7.5, but embryos are resorbed during organogenesis.	Rep, DDR, HDR*	Min et al., 2013
Lig3	E7.5	Embryos are smaller from gastrulation onset and do not develop past mid-gastrulation.	Rep, EJ, BER, NER	Puebla-Osorio et al., 2006
Ptip	E7.5	Gastrulating embryos display increased apoptosis. Embryos can be recovered in the early stages of organogenesis but are poorly formed.	Rep, DDR, EJ, HDR	Cho et al., 2003
Rad51B (Rec2)	E7.5	Knockout embryos implant, but do not develop pro-amniotic cavities and are resorbed by E7.5.	HDR	Shu et al., 1999
Uvrag	E7.5	Knockout mice die by E7.5 but no additional data is provided as to the cause of the lethality.	EJ*	Afzal et al., 2015
Wdr48 (Uaf1)	E7.5	Knockout mice die during gastrulation.	Rep, HDR	Park et al., 2013
Ercc3 (Xpb)	Before E8.5	No healthy embryos are recovered by early organogenesis. Embryos may be compromised earlier but no additional details are provided.	NER*	Andressoo et al., 2009
Ddb1	Before E8.5	Same as above.	CC, Rep, DDR, NER	Cang et al., 2006
Palb2	E8.5	Embryos are developmentally retarded after gastrulation and die during organogenesis.	Rep, HDR	Bowman-Colin et al., 2013
Rad51C	E8.5	Knockout embryos implant but are developmentally delayed, resulting in impaired gastrulation and resorption from E8.5.	Rep, HDR	Kuznetsov et al., 2009
Rev3L	After E8.5	Blastocysts form but do not thrive *in vitro*. Embryos are smaller than wildtype littermates during gastrulation.	HDR, TLS	Bemark et al., 2000
Pnkp	After E9.0	Embryos are not recovered from the onset of organogenesis. No further details are provided as to the cause of lethality.	EJ, BER	Shimada et al., 2015
Nipbl (Scc2)	Before E9.5	No knockout mice are recovered from the onset of organogenesis, but no additional data is provided.	Rep, DDR, HDR, EJ	Smith et al., 2014
Srpq	Before E9.5	Knockouts cannot be recovered from the start of organogenesis, implying that embryos die during gastrulation.	HDR*	Takeuchi et al., 2018
Ppp4C	Before E9.5	Same as above.	HDR	Shui et al., 2007
Rnaseh2B	From E9.5	Knockouts are smaller than littermates at gastrulation conclusion and die early in organogenesis.	Rep, DDR	Hiller et al., 2012
Syf2	E9.5	Knockouts do not survive organogenesis. Embryos can implant but gastrulation does not proceed normally.	CC, Rep	Chen et al., 2012
Nsmce2	Before E10.5	Knockout mice are not recovered from the uterus by E10.5. Embryos recovered at E2.5 appear normal but do not thrive *in vitro*. The cause of embryonic failure is not reported.	HDR	Jacome et al., 2015

*Key: (E) = Embryonic day; DDR = DNA damage response/damage sensing; CC = cell cycle control; Rep = DNA replication and/or replication stress; HDR = Homologous directed repair; EJ = End joining pathways; BER = Base excision repair; NER = Nucleotide excision repair; MMR = Mismatch repair; Telomere = telomere protein; TLS = Translesion synthesis. Gene functions are cross-referenced with the literature and may diverge from the MGI-GO categorization. Factors labeled with * indicate when the gene encodes a protein with additional functions outside genome stability that likely contribute to embryonic lethality.*

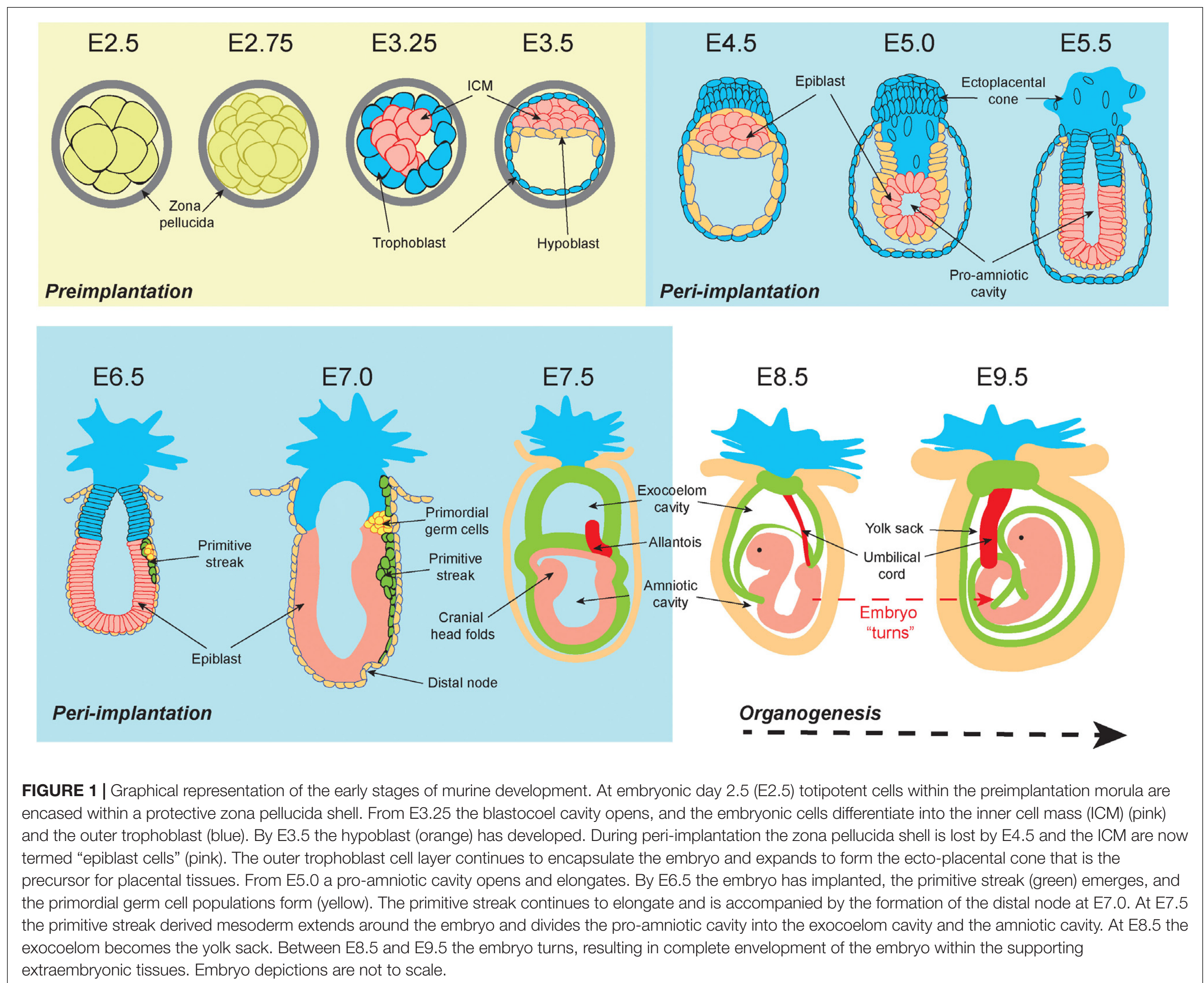

FIGURE 1 | Graphical representation of the early stages of murine development. At embryonic day 2.5 (E2.5) totipotent cells within the preimplantation morula are encased within a protective zona pellucida shell. From E3.25 the blastocoel cavity opens, and the embryonic cells differentiate into the inner cell mass (ICM) (pink) and the outer trophoblast (blue). By E3.5 the hypoblast (orange) has developed. During peri-implantation the zona pellucida shell is lost by E4.5 and the ICM are now termed "epiblast cells" (pink). The outer trophoblast cell layer continues to encapsulate the embryo and expands to form the ecto-placental cone that is the precursor for placental tissues. From E5.0 a pro-amniotic cavity opens and elongates. By E6.5 the embryo has implanted, the primitive streak (green) emerges, and the primordial germ cell populations form (yellow). The primitive streak continues to elongate and is accompanied by the formation of the distal node at E7.0. At E7.5 the primitive streak derived mesoderm extends around the embryo and divides the pro-amniotic cavity into the exocoelom cavity and the amniotic cavity. At E8.5 the exocoelom becomes the yolk sack. Between E8.5 and E9.5 the embryo turns, resulting in complete envelopment of the embryo within the supporting extraembryonic tissues. Embryo depictions are not to scale.

(nervous system and skin precursor), endoderm (gut precursor), and mesoderm (precursor for all other tissues) (Watson and Tam, 2001). Because continued gastrulation requires a critical mass of 1000 cells, delays in epiblast proliferation at E6.5 may result in the embryo forming a small primitive streak but developing no further (Snow and Tam, 1979; Tam, 1989; Power and Tam, 1993).

Between E6.5 and E7.5 the rate of epiblast proliferation is exceptionally pronounced, with some cell cycles completed in 2.2 h (Snow, 1977). Elongation of the primitive streak between E6.5 and E7.0 is associated with the first evidence of epithelial to mesenchymal transition (EMT) (Carver et al., 2001). EMT mobilizes epiblast cells to form the endoderm and mesoderm lineages, while cells that do not undergo EMT become the ectoderm (Acloque et al., 2009). At E7.5 the mouse embryo contains over 14,000 cells derived from epiblast progenitors. Proliferation slows at this point resulting in an increased average cell cycle duration of 8.1 h (Snow, 1977). There is evidence to suggest a that a specialized "proliferative zone" with sub 4-h cell cycles is maintained within the primitive streak after E7.5, however, definitive evidence remains to be substantiated (Tam et al., 2013).

Genome Instability Differentially Impacts Cell Viability in Pre- and Peri-Implantation Embryos

Embryonic lethality implies that one or more cell populations are compromised during development at the time of embryo failure. This may arise through DNA damage or genome instability, with multiple lines of evidence indicating that preimplantation embryos are more resistant to genome perturbations than peri-implantation embryos. For example, gamma irradiation-induced DNA damage at E6.5 or E7.5 confers apoptosis specifically within the epiblast (Heyer et al., 2000). Conversely, apoptosis levels are low in preimplantation embryos irradiated between E3.5 and E5.5, or during organogenesis from E8.5. It is therefore the epiblast that is particularly sensitive to these types of DNA lesions. Similarly, despite tetraploidy driving additional

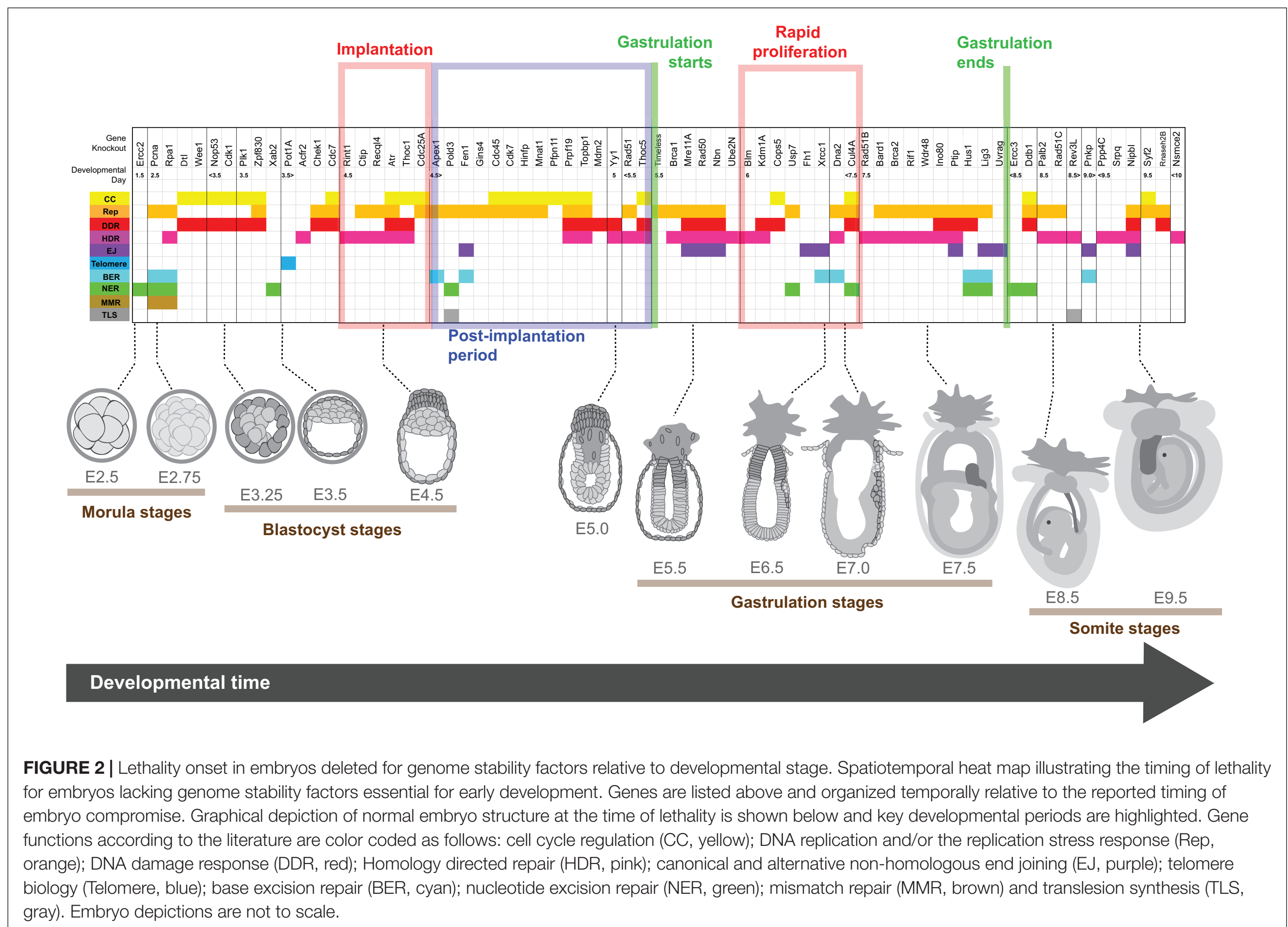

FIGURE 2 | Lethality onset in embryos deleted for genome stability factors relative to developmental stage. Spatiotemporal heat map illustrating the timing of lethality for embryos lacking genome stability factors essential for early development. Genes are listed above and organized temporally relative to the reported timing of embryo compromise. Graphical depiction of normal embryo structure at the time of lethality is shown below and key developmental periods are highlighted. Gene functions according to the literature are color coded as follows: cell cycle regulation (CC, yellow); DNA replication and/or the replication stress response (Rep, orange); DNA damage response (DDR, red); Homology directed repair (HDR, pink); canonical and alternative non-homologous end joining (EJ, purple); telomere biology (Telomere, blue); base excision repair (BER, cyan); nucleotide excision repair (NER, green); mismatch repair (MMR, brown) and translesion synthesis (TLS, gray). Embryo depictions are not to scale.

chromosomal instability in preimplantation cells (Paim and FitzHarris, 2019) chimeric mouse embryos with a mixture of diploid and tetraploid cells will develop through preimplantation before dying during peri-implantation (Horii et al., 2015). Notably, tetraploid sensitivity *in vivo* appears to be specific to the epiblast as embryos with tetraploid trophoblast cells and diploid epiblast cells can generate live pups (Wen et al., 2017). Mouse embryos containing a mixture of diploid and aneuploid cells will also develop to peri-implantation before the aneuploid cells are specifically depleted in the epiblast through apoptosis (Bolton et al., 2016).

As with somatic tissues, the tumor suppressor *TP53* (p53) plays a central role regulating stem cell outcomes following genomic insult. p53 orchestrates growth arrest or apoptosis following activation of the DNA damage response (Mello and Attardi, 2018). Concordantly, inhibiting p53-dependant signaling pathways enables chimeric embryos made from tetraploid preimplantation murine embryonic stem cells (mESCs) to survive until birth (Horii et al., 2015). Deleting *TP53* also reduced apoptosis levels in irradiated E6.5 embryos (Heyer et al., 2000) and extended the survival of embryos co-deleted for essential DNA repair factors (Jones et al., 1995; Haupt et al., 1997; Ludwig et al., 1997; Kim et al., 2002; McCarthy et al., 2003; Cang et al., 2006; Reinhardt and Schumacher, 2012). Not surprisingly, *TP53* was identified as a critical mediator of apoptosis in the gastrulating epiblast (Laurent and Blasi, 2015). However, when activated in pluripotent stem cells, p53 also influences the expression of pluripotency factors to regulate differentiation (Lin et al., 2005; Li et al., 2012; Akdemir et al., 2014; Jain et al., 2016). p53 therefore functions through canonical and unique pathways in early development to regulate cellular outcomes. This highlights that our classic understanding of genome stability pathways may not strictly apply to early development or certain pluripotent cell types (Zaveri and Dhawan, 2018).

DNA DAMAGE RESPONSE AND REPAIR PATHWAYS

Replication Stress Response

Somatic mammalian cells prepare for DNA replication in G1 phase by licensing replication origins and loading inactive Cdc45-MCM-GINS replicative helicase complexes (Bleichert, 2019; Miller et al., 2019). Cyclin dependent kinase activity promotes E2F transactivation to initiate replication

at the G1/S transition (Kent and Leone, 2019). Replication then proceeds throughout the S-phase with origins firing in temporal coordination and DNA synthesis occurring across the entirety of the genome (Burgers and Kunkel, 2017; Limas and Cook, 2019). Intrinsic and extrinsic factors may disrupt replication fork processivity: a phenomenon known as "replication stress" (Zeman and Cimprich, 2014). Replication stress is sensed through the accumulation of RPA binding to its single strand DNA (ssDNA) substrate (Bhat and Cortez, 2018). When replication stress stalls DNA synthesis the replicative helicase continues to unwind its substrate exposing ssDNA for RPA coating (Byun et al., 2005). ATR kinase is the master regulator of the replication stress response (Saldivar et al., 2017). RPA coated ssDNA recruits ATR and its associated protein ATRIP (Cortez et al., 2001) to stalled replication forks through parallel pathways mediated by TopBP1 and ETAA1 (Kumagai et al., 2006; Bass et al., 2016; Haahr et al., 2016). Once localized to the stalled fork, ATR is activated and propagates a signaling cascade resulting in engagement of the replication stress response. This includes activation of the downstream effector CHK1 kinase to arrest S phase until replication stress is resolved (Zhang and Hunter, 2014). During the replication stress response, stalled replication forks are often remodeled into a four-way structure and protected before engaging one of many diverse repair mechanisms dependent upon the underlying stress the fork encountered (Quinet et al., 2017; Cortez, 2019).

If replicative stress is unresolved, arrested replication forks may collapse into one-ended double strand breaks (DSBs) (Ait Saada et al., 2018). Additionally, persistent replication stress can result in under-replicated DNA persisting through S-phase, the second growth (G2) phase, and into the mitotic (M) phase of the cell cycle (Mankouri et al., 2013). Specialized repair mechanisms address replication defects carried into mitosis (Minocherhomji et al., 2015), during which time the canonical DSB repair pathways are inhibited (Orthwein et al., 2014). Replication defects passed into mitosis can confer chromosome segregation errors resulting in aneuploidy (Burrell et al., 2013; Wilhelm et al., 2019), or if severe mitotic death (Masamsetti et al., 2019). If a replication stressed cell escapes mitosis this is often evident in the daughter cells where the under-replicated DNA is present as a scar in the first growth phase (G1) that is repaired in the subsequent S phase (Lukas et al., 2011; Spies et al., 2019).

Double Strand Break Repair

DSBs are a major threat to genome stability because a failure in repair may result in loss of an entire chromosome arm (Scully et al., 2019). Additionally, chromosome segregation errors resulting from inadequate DSB repair are rife with implications for genome instability, including chromothripsis and kataegis (Maciejowski et al., 2015; Ly and Cleveland, 2017). DSBs are sensed by Ku70/80 (Gottlieb and Jackson, 1993; Ochi et al., 2015), PARP1 (Ali et al., 2012; Liu C. et al., 2017), and the MRN (MRE11, RAD50 and NBS1) complex (Lee and Paull, 2005; Stracker and Petrini, 2011). MRN facilitates recruitment of the ATM kinase to DSBs (Uziel et al., 2003) while Ku70/80 recruits a related kinase DNA-PKcs (Hnizda and Blundell, 2019). Once recruited to the break, ATM is activated and engages downstream DDR pathways (Dupre et al., 2006; Maréchal and Zou, 2013). This includes phosphorylation of the histone variant H2AX (termed γ-H2AX when phosphorylated) within the break adjacent chromatin and assembly of factors at the break locus to facilitate repair (Rogakou et al., 1998). ATM activation also results in growth arrest, including activation of the p53 pathway (Banin et al., 1998). Subsequent repair of DNA breaks is then orchestrated by homology directed repair (HDR) or end-joining (EJ) pathways.

Homology directed repair utilizes the homologous sister chromatid as a template for repair and is thus limited to the S and G2 cell cycle phases (Hustedt and Durocher, 2016). HDR initiates with resection at the broken DNA end to provide a 3′ ssDNA overhang for insertion into homologous regions of the sister chromatid (Symington, 2016). Once resection occurs at a canonical two-ended DSB, a series of enzymatic steps facilitate strand-invasion of the broken end into the sister chromatid, formation of a displacement-loop, template copying from the invaded DNA end, potential formation of a "Holliday Junction," and resolution of strand invasion (Scully et al., 2019). HDR factors also function within the replication stress response though diverse mechanisms including the remodeling and stabilization of stressed replication forks into four-way structures prior to repair (Neelsen and Lopes, 2015). Additionally, HDR factors participate in resolving one-ended DSBs that arise from collapsed replication forks to restart replication (Ait Saada et al., 2018). HDR is thus intrinsically linked to the replication stress response.

Conversely, EJ involves the covalent ligation of broken DNA ends to repair DSBs and thus does not require an additional chromatid template. End-joining mechanisms function in S and G2, but notably are the only DSB repair pathway available in G1 (Chang et al., 2017). Classical non-homologous end joining (c-NHEJ) directly ligates DNA ends, and if unregulated can also drive chromosome translocations (Ghezraoui et al., 2014) or telomere fusions (Celli et al., 2006; Van Ly et al., 2018). Cells can also engage alternative non-homologous end joining (alt-NHEJ), where DNA resection creates 3′ overhangs to align the DNA ends for repair through microhomology (Sfeir and Symington, 2015). This often requires a fill-in reaction by the error prone polymerase Pol θ which can introduce errors into the repaired sequence (Mateos-Gomez et al., 2015; Mateos-Gomez et al., 2017).

Nucleotide and Base Damage

In addition to DNA breaks, cells must also contend with damage to nucleotide bases, base mismatches, and bulky DNA lesions that distort the DNA double helix. Base excision repair (BER) mends small non-distorting DNA lesions such as oxidized DNA bases (Wallace, 2014), while nucleotide excision repair (NER) corrects bulky lesions such UV-induced pyrimidine dimers (Schärer, 2013). BER is mediated by DNA

glycosylases that cleave and remove the damaged bases at the lesion. A correct base is then inserted by specialized DNA polymerases (Wallace, 2014). In NER, a 25–30 nucleotide patch of ssDNA containing the bulky lesion is excised from the double strand helix before specialized DNA polymerases fill in the ssDNA gap (Schärer, 2013). Mismatch repair (MMR) proofreads the newly replicated DNA to identify mis-incorporated bases (Liu D. et al., 2017). When a mismatch is identified, the newly synthesized strand is nicked and resected creating a patch of exposed ssDNA for fill in by a high-fidelity DNA polymerase.

Essentiality of DNA Repair During Embryogenesis

Not all DNA repair activities are essential for early development (**Supplementary Table S1**). The most striking example is the dichotomy between the major DSB repair pathways. While many HDR factors are essential, the core c-NHEJ factors are either dispensable for development [*Ku70* (Ouyang et al., 1997), *Ku80* (Nussenzweig et al., 1996; Gu et al., 1997), *Prkdc* (DNA-PKcs) (Kurimasa et al., 1999), *Nhej1* (XLF) (Li et al., 2008)] or only required for organogenesis [*Lig4* (Frank et al., 1998), *Xrcc4* (Dickinson et al., 2016)]. Some alt-NHEJ components are required for early development. Alt-NHEJ is promoted by the polymerase Pol θ which primes the DNA ends for repair by removing RPA from exposed ssDNA regions (Kent et al., 2015; Mateos-Gomez et al., 2017). Alt-NHEJ is also dependent on PARP-1, XRCC1 and LIG3 (Audebert et al., 2004), with PARP-1 having a critical role in synapsis, and XRCC1 and LIG3 mediating DNA ligation (Audebert et al., 2004). While *Lig3* and *Xrcc1* knockout embryos die during gastrulation (Tebbs et al., 1999; Puebla-Osorio et al., 2006), both genes encode factors that have functions in other DNA metabolic activities outside EJ (Wallace, 2014). Conversely *Pol* θ knockout mice are viable (Shima et al., 2004; Masuda et al., 2005), as are *Parp1* knockouts (Wang et al., 1995), suggesting that alt-NHEJ is not essential for embryogenesis.

Preference for HDR over NHEJ is further evident in mechanistic studies of DDR engagement within embryonic stem cells (Tichy et al., 2010). Following DSB induction, ATM-dependent γ-H2AX phosphorylation at the break locus recruits the scaffolding protein MDC1 (Stewart et al., 2003). MDC1 subsequently recruits the ubiquitin ligases RNF8 and RNF168 that signal downstream recruitment of 53BP1 (Jackson and Durocher, 2013). 53BP1 then establishes a physical domain at the repair locus in coordination with RIF1 to inhibit end resection and HDR in favor of c-NHEJ (Chapman et al., 2013; Zimmermann et al., 2013; Lou et al., 2019; Ochs et al., 2019). Notably, many factors that promote c-NHEJ are dispensable for embryonic development, including *Atm* (Barlow et al., 1996; Xu et al., 1996), *H2ax* (Celeste et al., 2002), *Mdc1* (Lou et al., 2006), *Rnf8* (Valnegri et al., 2017), *Rfn168* (Zong et al., 2019), and *Trp53bp1* (53BP1) (Ward et al., 2003). Additionally, multiple reports demonstrate that 53BP1 does not localize to DSB foci in the ICM of preimplantation embryos or mESCs grown in culture (Ziegler-Birling et al., 2009; Kafer et al., 2016). *In vivo*, 53BP1 localizes to damage in E5.5 embryos, but only in the epiblast cells and not the endoderm or trophoblast cells (Laurent and Blasi, 2015). While *Trp53bp1* expression is unchanged in early development, *Rnf168* expression is limited prior to the epiblast stage which may explain differential engagement of DDR pathways in pre- and peri-implantation cells (Laurent and Blasi, 2015).

GENES ESSENTIAL FOR PREIMPLANTATION DEVELOPMENT

Consistent with a greater tolerance of genome instability in preimplantation cells, only 10 knockouts of genome stability factors that we reviewed were lethal during preimplantation (**Table 1** and **Supplementary Table S1**). Factors encoded by many of these preimplantation essential genes also possessed critical functions outside DDR or repair. Additionally, preimplantation embryos commonly survive to the morula stage even if a targeted gene is essential for viability. This is because embryonic genomes are not immediately active following fertilization while the inherited maternal mRNA guides protein translation (Jukam et al., 2017).

Multifunctional Factors Essential for Preimplantation Development

Of the 10 genome stability factors required for preimplantation development, only *Ercc2* deletion did not permit morula generation. *Ercc2*$^{-/-}$ embryos fail to develop past the 2-cell stage *in vitro* and there is no evidence of embryo implantation *in vivo* (de Boer et al., 1998). *Ercc2* encodes the XPD helicase that functions both in NER and as a core component of the general transcription factor IIH (TFIIH) complex. TFIIH opens DNA and anchors the cdk-activating kinase (CAK) complex to facilitate transcription (Egly, 2001). Given that *Ercc2* knockouts are compromised during embryonic genome activation (EGA), a reasonable conclusion is that preimplantation lethality is associated with a loss of XPD transcriptional activity. Likewise, *Xab2* is a TFIIH and NER component and *Xab2* deletion is lethal prior to the blastocyst stage (Yonemasu et al., 2005). However, *Xab2* null embryos survive past the 2-cell stage suggesting *Xab2* is not required for EGA.

Pcna and *Rpa1* both function in multiple genome maintenance pathways and deleting either gene confers preimplantation lethality (Wang et al., 2005; Roa et al., 2008). PCNA (proliferating cell nuclear antigen) forms a homotrimeric sliding clamp that encircles DNA. The protein clamp travels with the replication fork to promote replicative polymerase processivity and mediate diverse functions in DNA replication and the replication stress response (Mailand et al., 2013). PCNA also functions in NER and MMR (Strzalka and Ziemienowicz, 2011). *Rpa1* (Replication Protein A 1) codes

for the 70 kDa subunit of the heterotrimeric RPA ssDNA binding complex that participates in replication, but which also functions in ATR activation, replication fork repair, HDR and NER (Bhat and Cortez, 2018). Analysis of *Pcna* mRNA showed a significant reduction of maternal transcripts from the zygote to the 2-cell stage consistent with the observed early embryonic lethality (Hamatani et al., 2004; Roa et al., 2008; Bult et al., 2019). *Rpa1* null embryos form blastocysts at E3.5 that are smaller than their heterozygous littermates and which fail in blastocyst outgrowth assays, indicating *Rpa1* is required for trophectoderm and ICM growth (Wang et al., 2005). While both *Rpa1* and *Pcna* function in diverse aspects of genome maintenance, both genes are also required for DNA replication. Preimplantation failure of *Pcna* or *Rpa1* null embryos likely stems from an inability to polymerize nascent DNA coupled with simultaneous attenuation of multiple DNA repair pathways.

Two additional genes classified as DNA repair factors in the MGI-GO database are also essential during preimplantation. *Nop53* encodes a nucleolar protein with suggested roles regulating p53, DNA repair, and the cellular response to mitochondrial stress (Lee et al., 2012; Yoon et al., 2014). *Nop53* null embryos fail to develop past the blastocyst stage, while *Nop53*$^{-/-}$ mESCs are viable, suggesting that *Nop53* functions in morula to blastocyst maturation (Sasaki et al., 2011). In somatic cells, *Pot1a* encodes a telomere specific ssDNA binding protein that suppresses ATR and p53 activation by the chromosome end (Hockemeyer et al., 2005, 2006; Denchi and de Lange, 2007). Loss of *Pot1a* likely confers robust ATR activation which explains the failure of *Pot1a*$^{-/-}$ embryos to form an ICM (Hockemeyer et al., 2006).

Cell Cycle and Checkpoint Factors Essential for Preimplantation Viability

In the event of replication stress or DSBs, checkpoints slow the cell cycle to provide time for DNA repair. Deletion of several genes that encode checkpoint regulatory factors confer preimplantation embryo failure at the morula stage. These include *Wee1* (Tominaga et al., 2006), *Cdk1* (Diril et al., 2012), and *Plk1* (Lu et al., 2008; Wachowicz et al., 2016). CDK1 promotes the G2/M transition through interaction with cyclin B, and CDK1-cyclin B activity is regulated by WEE1 (Harvey et al., 2005; Gavet and Pines, 2010). ATR and CHK1 activate the G2/M checkpoint by stimulating WEE1 to inhibit CDK1 (O'Connell et al., 1997; Nigg, 2001). Polo-like kinase 1 (PLK1) controls numerous mitotic activities, participates in the G2/M transition, may function in DNA replication, and is implicated in HDR through phosphorylation of RAD51 (Watanabe et al., 2004; Mandal and Strebhardt, 2013; Yata et al., 2014; Pintard and Archambault, 2018). *Dtl* is also required for preimplantation development (Liu et al., 2007). *Dtl* encodes a component of the CUL4A-DDB1 E3-ubiquitin ligase complex that degrades the replication licensing factor CDT1 to stimulate replication and S phase progression (Nishitani et al., 2001; Li and Blow, 2005). Because these essential cell cycle genes function in DDR-regulated checkpoints and normal cell cycle transitions, the mechanism of embryonic lethality following their deletion likely involves deregulation of both the DDR and normal cell cycles.

GENES ESSENTIAL FOR PERI-IMPLANTATION DEVELOPMENT

Peri-implantation is the period of embryonic development most associated with lethality following deletion of genome stability factors. Within the MGI-GO derived gene list, it stands out that 54 of the 62 genes essential for peri-implantation development have direct or indirect functions in DNA replication, the replication stress response, and/or HDR (**Table 2** and **Supplementary Table S1**). Below we focus specifically on these pathways.

Genes Regulating Replication Initiation, DNA Polymerization, and the Replication Stress Response Are Essential for Peri-Implantation Development

Because of the rate of cell proliferation in peri-implantation embryos, it is perhaps unsurprising that factors which regulate the G1/S transition and initiate DNA replication are essential for peri-implantation development. This includes *Cdc7* (Kim et al., 2002), *Cdc45* (Yoshida et al., 2001), *Cdk7* (Ganuza et al., 2012), *Mnat1* (Rossi et al., 2001), *Gins4* (Mohri et al., 2013), *Cul4A* (Li et al., 2002), and *Ddb1* (Cang et al., 2006). Additionally, the *Pold3* subunit of the replicative polymerase *Pol*δ is required for peri-implantation development (Uchimura et al., 2009; Zhou et al., 2018), as is *Fen1*, which plays a central role in processing Okazaki fragments on the lagging strand of DNA synthesis (Larsen et al., 2003). Lethality following deletion of most of these genes occurs on or near E4.5 and is associated with proliferative failure within the ICM after blastocyst formation but prior to gastrulation. A likely explanation is that peri-implantation development is rapidly compromised when fundamental functions necessary for cellular proliferation and/or DNA replication are dysregulated.

Even in the absence of exogenous threats to replication, the exceptionally fast cell cycles within the developing epiblast are expected to confer intrinsic replication stress. For example, blastocyst derived mESCs display replication stress in unperturbed cultures *in vitro* (Ahuja et al., 2016). mESCs appear to manage this replication stress by effectively coupling replication and repair activities to facilitate near continuous DNA synthesis punctuated by brief G1 and M phases (Ahuja et al., 2016). Epiblast cell cycles *in vivo* are more rapid than cell cycles in cultured mESCs (Snow, 1977; Mohammed et al., 2017) suggesting that a similar reliance on efficient coupling of DNA replication and the repair activity is required to manage replication stress in peri-implantation embryos.

The replication stress response begins when ATR/ATRIP localizes to RPA-coated ssDNA, and ATR is activated through

TOPBP1 and the RAD9-RAD1-HUS1 (9-1-1) protein complex (Kumagai et al., 2006; Navadgi-Patil and Burgers, 2009; Choi et al., 2010). 9-1-1 is a sliding clamp loaded onto DNA (Bermudez et al., 2003). TOPBP1 bridges ATR/ATRIP and 9-1-1, thereby stabilizing ATR at the fork. Once stabilized at the site of replication stress, ATR regulates downstream replication stress repair and cell cycle arrest via CHK1 kinase and CDC25A (Liu et al., 2000; Xiao et al., 2003; Delacroix et al., 2007). TIMELESS, a component of the replication fork protection complex (Kemp et al., 2010; Couch et al., 2013; Buisson et al., 2017), and the ubiquitin ligase PRP19 (*Prpf19*) (Maréchal et al., 2014), also function in ATR and CHK1 activation. Embryos null for *Atr* (Brown and Baltimore, 2000; de Klein et al., 2000; Murga et al., 2009), *Topbp1* (Jeon et al., 2011), *Hus1* (Weiss et al., 2000), *Chek1* (CHK1) (Takai et al., 2000; Zaugg et al., 2007), *Cdc25A* (Ray et al., 2007), *Timeless* (Gotter et al., 2000), and *Prpf19* (Fortschegger et al., 2007) are all compromised during peri-implantation.

In somatic tissues, ATR or CHK1 inhibition coupled with replication stress induces replication catastrophe and S-phase apoptosis (Myers et al., 2009; Toledo et al., 2013; van Harten et al., 2019). Similarly, *Atr* null embryos form blastocysts that *in vitro* display widespread apoptosis within the ICM but not in the trophoblast, and which are eventually compromised by day three of culture (Brown and Baltimore, 2000; de Klein et al., 2000; Murga et al., 2009). ATR activity is therefore essential in the pluripotent and rapidly proliferating epiblast cells that will become the embryo proper, but not in the multipotent placental precursors. *Chek1* null embryos also form blastocysts, but unlike *Atr* null embryos, are unable to attach to the culture vessels in outgrowth experiments (Takai et al., 2000). The more severe embryonic response to a loss of CHK1 activity compared to ATR inhibition is consistent with observations in somatic cells (Buisson et al., 2015).

HDR Genes Are Essential for Peri-Implantation Development

Common HDR factors manipulate DNA substrates at two-ended DNA breaks and stressed replication forks (Ait Saada et al., 2018). During replication stress, this entails regressing and protecting stalled forks in 4-way DNA structures to facilitate repair (Quinet et al., 2017). HDR factors essential for peri-implantation that function in canonical DSB repair and replication fork protection include: *Brca1* (Hakem et al., 1996; Ludwig et al., 1997; Taglialatela et al., 2017), *Brca2* (Sharan et al., 1997; Lemaçon et al., 2017; Mijic et al., 2017), *Rad51* (Lim and Hasty, 1996; Tsuzuki et al., 1996; Zellweger et al., 2015), *Bard1* (McCarthy et al., 2003; Shakya et al., 2008; Daza-Martin et al., 2019), *Palb2* (Bowman-Colin et al., 2013), and *Rad51C* (Kuznetsov et al., 2009; Somyajit et al., 2015). Similarly, common factors promote resection during HDR repair of canonical two-ended DSBs and at stalled replication forks (Ait Saada et al., 2018). This includes the essential peri-implantation genes: *Ctip* (Chen et al., 2005; Przetocka et al., 2018); *Ptip* (Cho et al., 2003; Ray Chaudhuri et al., 2016); *Dna2* (Lin et al., 2013; Thangavel et al., 2015), and the MRN complex [*Mre11*, *Rad50*, and *Nbs1* (Xiao and Weaver, 1997; Luo et al., 1999; Zhu et al., 2001; Theunissen et al., 2003; Buis et al., 2008; Schlacher et al., 2011).

Homology directed repair activity in replication fork remodeling and protection is an emerging topic in the DNA repair field, and it remains unclear which HDR functions are essential for cell viability. However, lethality in *Brca2* null mESCs is rescued when fork protection is facilitated by inhibiting PARP activity to protect replication forks from MRE11 nuclease (Ding et al., 2016). For BRCA2, fork protection may be the essential function. *In vivo*, deleting factors that participate in both canonical HDR-dependent DSB repair and fork remodeling or resection, typically confers reduced cell proliferation and embryo failure during gastrulation. For example, *Brca1*$^{-/-}$ embryos are severely malformed with an underdeveloped pro-amniotic cavity and mesoderm (Hakem et al., 1996; Ludwig et al., 1997). Concordantly, *Brca1* null embryos display a significant reduction of DNA synthesis by E6.5, increased p21 [a factor induced by p53 transactivation to mediate cell cycle arrest (Georgakilas et al., 2017)], and a high incidence of G1 arrested cells (Hakem et al., 1996). Similarly, *Rad51* null embryos suffer from reduced proliferation and begin to degenerate during gastrulation by E7.5, but development can be extended to E9.5 in *TP53/Rad51* double knockouts (Lim and Hasty, 1996). Loss of HDR factors therefore appears to primarily confer proliferative failure through p53-induced growth arrest and subsequently through accumulated genome instability if p53 function is compromised.

Additional Peri-Implantation Genes Linked to HDR or Replication

Peri-implantation development additionally requires several genes that are functionally linked to HDR or replication stress mitigation. Such genes include *Apex1* (Xanthoudakis et al., 1996), *Rnaseh2B* (Hiller et al., 2012), *Rif1* (Chapman et al., 2013), *Blm* (Chester et al., 1998), *Rev3L* (Bemark et al., 2000), *Usp7* (Kon et al., 2010), and *Ino80* (Min et al., 2013). *Apex1* does not directly function in replication, but is critical for the repair of abasic DNA sites that if left unresolved confer replication stress (Boiteux and Guillet, 2004). Similarly, *Rnaseh2B* resolves RNA within RNA-DNA hybrids, including mis-incorporated ribonucleotides, which if not resolved can impede replication (Pizzi et al., 2015). In addition to functioning in DSB repair pathway choice, *Rif1* also coordinates replication timing (Yamazaki et al., 2012; Foti et al., 2016). *Blm* encodes a helicase that functions in homologous recombination, the replication stress response (Davies et al., 2007; Machwe et al., 2011), promotes replication at telomeres (Barefield and Karlseder, 2012), and participates in a specialized form of break induced replication at chromosome ends (Sobinoff et al., 2017). *Rev3L* encodes the catalytic subunit of POL ζ which facilitates translesion synthesis to enable replication forks to negotiate lesions within DNA without stalling (Bhat et al., 2013).

Usp7 is a deubiquitylating enzyme that promotes efficient DNA replication (Lecona et al., 2016) and *Ino80* encodes a chromatin remodeling factor that functions in both replication and HDR (Poli et al., 2017).

MAINTAINING GENOME STABILITY DURING PERI-IMPLANTATION DEVELOPMENT

While HDR and replication associated genes are not the only genome stability factors required for peri-implantation development, their predominance on the list of genes essential for this developmental window is compelling. Conditions enabling *in vitro* culture of peri-implantation murine epiblast stem cells are now established (Brons et al., 2007; Tesar et al., 2007). However, there are no published studies focusing on genome maintenance in isolated epiblast cells. It is thus necessary to extrapolate from studies of other rapidly dividing cells why HDR and replication factors may be essential during peri-implantation development.

Replication Stress in Peri-Implantation Cells

The fastest cell cycles in the lifetime of a mouse are likely during the brief window of epiblast development. Outside of the embryo, rapid proliferation contributes to replication stress in hematopoietic and cancerous tissues by robbing cells of the time to properly execute DNA polymerization (Pilzecker et al., 2017). For example, during a somatic G1-phase replication is facilitated by increasing the abundance of MCM proteins and loading these factors at replication origins in preparation for S-phase (Boos et al., 2012). Late G1 cells also upregulate dNTP production for the oncoming burst of DNA synthesis (Hirschi et al., 2010; Dick and Rubin, 2013). When origins are not established properly, or dNTP production is dysregulated, cancer and aging hematopoietic cells are susceptible to replication stress (Flach et al., 2014; Aird and Zhang, 2015; Alvarez et al., 2015; Garzon et al., 2017).

Pluripotent cells in the early embryo display almost non-existent G1 phases and appear to use unique mechanisms to ensure S-phase progression is not impeded. For example, human ESCs counter G1 brevity by loading MCM proteins faster than cultured non-pluripotent cells (Matson et al., 2017). Retinoblastoma protein is also constitutively hyperphosphorylated in mouse and human ESCs (Conklin et al., 2012; Ter Huurne et al., 2017), which maintains high E2F transactivation to promote unperturbed dNTP synthesis (Hirschi et al., 2010; Dick and Rubin, 2013). To ensure rapid S-phase progression, mouse ESCs also license more dormant origins than non-embryonic progenitor cells (Ge et al., 2015). This increases the ability of ESCs to complete replication during a short S phase (Courtot et al., 2018). ESCs also demonstrate an increased ability to restart stalled replication forks (Ahuja et al., 2016; Zhao et al., 2018). Human induced pluripotent stem cells (iPSC) also license replication origins rapidly in their abbreviated G1 phase (Matson et al., 2017), and dormant origins are critical to maintain rapid proliferation in hematopoietic stem cell pools (Alvarez et al., 2015). Although not yet directly tested, we anticipate that epiblast cells employ similar countermeasures to expedite DNA synthesis.

Despite these efforts, human and mouse ESCs display endogenous replication stress in unperturbed cultures (Ahuja et al., 2016; Lamm et al., 2016). It is not clear why replication is stressed in pluripotent cells, however in proliferating cancer cells transcription commonly drives replication stress through collisions between RNA and DNA polymerases, or persistent RNA/DNA hybrid molecules termed R-loops (Crossley et al., 2019). Primary somatic cells mitigate transcription and R-loop interference by temporally coordinating transcription and replication during S-phase (Meryet-Figuiere et al., 2014). It is probable that peri-implantation epiblast cells facilitate rapid genome duplication by simultaneously engaging large numbers of origins. Such activity could increase the probability of replisome and transcriptome collisions and/or R-loop induced replication stress.

Under-replication due to endogenous stress is described in mESCs and human iPSCs (Ahuja et al., 2016; Vallabhaneni et al., 2018). Lengthening the G1 phase reduces replication defects in mESCs consistent with the notion that replication stress is driven by rushed cell cycles (Ahuja et al., 2016). Pluripotent cells are reported to have enhanced DNA repair capacities and it is possible this translates to highly effectively management of inefficient replication in embryos (Maynard et al., 2008; Luo et al., 2012). Alternatively, pluripotent blastocyst cells persist for a limited number of cell divisions *in vivo*, and it is feasible that pre-implantation embryos simply tolerate under-replication for this brief period. Tolerance of replication defects is consistent with observations that preimplantation embryonic cells are more resistant to genomic insult than peri-implantation cells. Compromising the replication stress response should confer similar DDR activation, chromosomal segregation errors, and aneuploidy in both pre- and peri-implantation cells. However, cells with supernumerary chromosomes persist in the blastocyst before elimination during peri-implantation (Bolton et al., 2016). This may explain why many genome stability factors become essential specifically during peri-implantation development. *In vivo,* activating p53 through deletion of its negative regulator *Mdm2* leads to lethality during peri-implantation (Jones et al., 1995). This suggests that p53 signaling during peri-implantation is a conduit to remove genomically unstable cells *in vivo*.

Additionally, it is important to recognize that genome stability factors may possesses cryptic essential functions during early development. For example, PARP1 is DDR factor involved in multiple aspects of genome integrity (Ray Chaudhuri and Nussenzweig, 2017). However, in mESCs, *Parp1* has roles preventing the *trans*-differentiation of extraembryonic trophoblast cells (Nozaki et al., 1999; Hemberger et al., 2003). This pluripotency-specific function is linked to the DNA binding ability of PARP1 which confers an epigenetic-like regulation of pluripotency (Roper et al., 2014). Additionally, pluripotency-specific repair factors may provide essential

functions in early development. The Filia-Floped protein complex is abundantly expressed in pluripotent but not somatic cells, and is suggested to increase the abundance of essential repair factors including BLM and promote ATR activation to encourage fork restart (Zhao et al., 2015; Zhao et al., 2018; Zheng, 2020).

Essential Roles for HDR Factors During Peri-Implantation

It is possible the canonical G1-phase c-NHEJ DSB repair pathway is dispensable during peri-implantation because cells rapidly transition through their brief G1 phase and engage alt-NHEJ or HDR in S-phase. Conversely, c- and alt-NHEJ remain active in HDR-deficient embryos, and in principle EJ functions could provide redundant DSB repair activity. An explanation why c- or alt-NHEJ cannot rescue HDR during peri-implantation may stem from the diversity of substrates repaired by HDR. While two-ended DSBs are readily repaired by EJ, collapsed replication forks present as one-ended DNA breaks which c- or alt-NHEJ cannot process (Feng and Jasin, 2017; Scully et al., 2019). One-ended breaks, however, can be repaired through a specialized HDR mechanism termed break-induced replication (BIR). During BIR, the exposed DNA end is resected, and following strand-invasion to form a displacement loop on the sister chromatid, DNA is polymerized from the invaded strand in a conservative manner (Kramara et al., 2018). While BIR is commonly studied in yeast, the analogous mechanism in vertebrates remains poorly defined. Notwithstanding, *Blm* promotes BIR at chromosome ends in human cancer cells that maintain telomere length through the alternative lengthening of telomeres mechanism suggesting that *Blm* may promote BIR elsewhere in the genome following replication stress (Sobinoff et al., 2017). Additionally, *Pold3* is also implicated in vertebrate BIR (Costantino et al., 2014). Conversely, the non-essential gene *Rad52* also functions in vertebrate BIR casting doubt on the requirement for BIR in early development and indicating the need for future studies (Sotiriou et al., 2016).

Multiple HDR factors essential for peri-implantation also play central roles in replication stress management. This includes HDR factors that regress, stabilize, or resect stalled replication forks including *Brca1/2, Bard1, Rad51* and *Rad51C*, *Ctip, Ptip, Dna2*, and genes encoding the MRN complex. Interestingly, the helicases and translocases that promote fork regression, including *Smarcal1*, are not essential for peri-implantation (Baradaran-Heravi et al., 2012). This may reflect redundancy in the mechanisms that drive formation of 4-strand structures at stalled replication forks (Quinet et al., 2017). Given the essentiality of the replication stress response for epiblast development, it stands to reason that replication fork remodeling and protective functions encoded by HDR factors play a role in the viability of peri-implantation embryos. Failure to facilitate these activities would confer genome instability, chromosome segregation errors, and molecular outcomes consistent with unrepaired replication stress. As described above, this would activate p53 surveillance systems during peri-implantation. In the future it will be interesting to determine which specific HDR functions are essential for embryogenesis.

CONCLUSION AND FUTURE DIRECTIONS

While most DDR and repair factors are non-essential, this does not exclude non-essential genome stability pathways from playing an important role in healthy development. Deletion of non-essential DDR and repair genes commonly results in the birth of live pups exhibiting a wide array of deleterious phenotypes (**Supplementary Table S1**). Additionally, cancer predisposition and a reduced lifespan are common in mice lacking non-essential genome stability factors (**Supplementary Table S1**). The difference between essential and non-essential genome stability genes is likely derived from the necessity of the targeted repair pathway to resolve lethal genome instability that arises during a specific window of development.

The critical genome stability pathways required for cell survival are determined by the type of genomic lesions the cell encounters and when in the cell cycle those lesions arise. Early embryonic development is largely protected from exogenous influence. Preimplantation embryos are wholly contained, and maternal blood supply begins at E9.5 (Behringer et al., 2014). The critical early stages of development likely benefit from sequestration from external threats to genome stability. We suggest the imminent threat to genome stability in early development stems from the need to mitigate endogenous replication stress within the epiblast to sustain rapid cell cycles. If left unresolved, replication stress drives genome instability, chromosome segregation errors, growth arrest and/or cell death. Dwindling cell numbers of epiblast cells that fail to effectively replicate their genome will progressively lead to embryo compromise. This premise is supported by the timing of embryo failure associated with deletion of essential DDR and repair genes. Consistent with systemic proliferative failure, deletion of factors required for basal DNA replication, or up-stream signaling in the replication stress response, typically induce an early embryonic demise (E5.0 or before). Whereas deleting HDR factors induces death at a subsequent time (E5.5 or later), potentially as the additive outcome of progressive genome instability.

Another caveat of reports reviewed here is that many were pioneering studies that utilized the newly developed technology of gene targeting in mice. In the intervening decades, our understanding of early development and experimental capability has blossomed. Development of new stem cell culture technologies now enables mechanistic study of peri-implantation development within three-dimensional gastrula structures *in vitro*. Co-culture of ICM derived ESCs, trophectoderm derived trophoblast stem cells, and extraembryonic endoderm stem cells results in the spontaneous self-assembly of a structure remarkably like the gastrulating embryo. These structures, termed "ETX embryos" will enable directed gene deletion within specific embryonic cell types (Sozen et al., 2018). Coupled with precise mechanistic investigations, ETX embryo models will reveal new insights on the mechanisms of cell compromise

during embryogenesis. It will be exciting to learn in the coming years why a limited number of DDR and repair pathways are essential for development, and the underlying reasons for their importance within the peri-implantation embryo.

DATA AVAILABILITY STATEMENT

MGI-GO designations discussed in this review can be found at the following. DNA damage checkpoint: http://www.informatics.jax.org/go/term/GO:0000077; nucleotide excision repair: http://www.informatics.jax.org/go/term/GO:0006289; mismatch repair: http://www.informatics.jax.org/go/term/GO:0006298; base excision repair: http://www.informatics.jax.org/go/term/GO:0006284; DSB repair via homologous recombination: http://www.informatics.jax.org/go/term/GO:0000724; DSB repair via non-homologous end joining: http://www.informatics.jax.org/go/term/GO:0006303.

AUTHOR CONTRIBUTIONS

GK and AC conceived of the review manuscript. GK compiled the information presented in **Tables 1**, **2** and **Supplementary Table S1** from the MGI-GO database as indicated. GK made the figures. GK and AC composed the manuscript.

FUNDING

The Cesare laboratory is supported by grants from the Australian National Health and Medical Research Council (1162886 and 1185870) and The Neil and Norma Hill Foundation.

ACKNOWLEDGMENTS

The authors thank members of the Cesare and Tam laboratories at the Children's Medical Research Institute for their helpful discussion.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.00416/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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