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Review

Role of zinc in female reproduction

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Abstract

Zinc is a critical component in a number of conserved processes that regulate female germ cell growth, fertility, and pregnancy. During follicle development, a sufficient intracellular concentration of zinc in the oocyte maintains meiotic arrest at prophase I until the germ cell is ready to undergo maturation. An adequate supply of zinc is necessary for the oocyte to form a fertilization-competent egg as dietary zinc deficiency or chelation of zinc disrupts maturation and reduces the oocyte quality. Following sperm fusion to the egg to initiate the acrosomal reaction, a quick release of zinc, known as the zinc spark, induces egg activation in addition to facilitating zona pellucida hardening and reducing sperm motility to prevent polyspermy. Symmetric division, proliferation, and differentiation of the preimplantation embryo rely on zinc availability, both during the oocyte development and post-fertilization. Further, the fetal contribution to the placenta, fetal limb growth, and neural tube development are hindered in females challenged with zinc deficiency during pregnancy. In this review, we discuss the role of zinc in germ cell development, fertilization, and pregnancy with a focus on recent studies in mammalian females. We further detail the fundamental zinc-mediated reproductive processes that have only been explored in non-mammalian species and speculate on the role of zinc in similar mechanisms of female mammals. The evidence collected over the last decade highlights the necessity of zinc for normal fertility and healthy pregnancy outcomes, which suggests zinc supplementation should be considered for reproductive age women at risk of zinc deficiency.

Summary sentence

An overview of the recent discoveries on the role of zinc in the reproductive processes of mammalian females including oogenesis, folliculogenesis, ovulation, maturation, fertilization, and pre- and post-implantation development.

Key words: zinc, mammal, female.

Introduction

Zinc is an essential nutrient involved in a multitude of physiological processes necessary for growth and survival. As a transition metal, zinc is indispensable for the catalytic activity of hundreds of enzymes, including two that are key for DNA synthesis: thymidine kinase and DNA polymerase [1, 2]. An estimated 3000 proteins bind zinc to maintain structural integrity and function, such as the major ribosomal RNA-transcribing enzyme, RNA polymerase I [3, 4]. Epigenetic reprogramming through histone and DNA methylation, metal-response element-regulated gene expression, and chromatin compaction are all regulated by the concentration of free intracellular zinc [5–9]. Additionally, transient changes in the intracellular zinc content and zinc efflux into the local extracellular space are used as intra- and intercellular signaling mechanisms in various cells and

tissues [10–13]. For instance, zinc is co-stored and co-released with insulin in beta cells to mediate autocrine and paracrine signaling, and altered zinc homeostasis in the pancreas is associated with diabetes [14].

As zinc plays an integral role in cell physiology and biochemistry, zinc levels are tightly regulated at the cellular level. In general, cellular zinc is present in one of two pools: (1) zinc that is tightly bound to proteins, such as zinc finger proteins, to maintain the protein structure and stability and where zinc is relatively unavailable for the other cellular functions (for review, see [15]) or (2) labile zinc that is sequestered in the cytoplasm by proteins, such as metallothionein, or in various intracellular compartments, including mitochondria and secretory vesicles. Labile zinc is readily accessible for cellular signaling and other physiological processes. Twenty-four mammalian zinc transporters have been identified, which are expressed across cytoplasmic and organelle membranes and move zinc in and out of various compartments. Of these transporters, the zinc transporter (ZnT), or solute carrier 30 (Slc30) family, consists of 10 proteins that control efflux from the cytoplasm to either the extracellular space or into organelles. A second family, the Zrt- and Irt-like protein (Zip), or solute carrier 39A (Slc39a) family, consists of 14 transporters that control influx into the cytoplasm from organelle stores or the extracellular milieu. These zinc transporters are reviewed extensively in [16–18].

Mammals lack an inherent tissue that can act as a zinc reserve to store or supply zinc in response to zinc availability in the diet, therefore, an adequate and regular intake of dietary zinc is necessary to balance the loss through excretion and to maintain normal zinc homeostasis [19]. The recommended daily intake of zinc ranges from 4.7 to 18.6 mg, depending on sex, age, and other physiological factors [20]. Individuals from low-income regions, particularly children, women of reproductive age, and the elderly, have an elevated risk of zinc deficiency (ZD) [20, 21]. Worldwide, an estimated one in seven to one in five people are at risk of ZD [22, 23]. Even in high-income regions, ZD is estimated to affect 7.5% of the population and may affect up to 30% of the population in poorer regions [22, 23].

Given the many roles for cellular zinc, it is not surprising that ZD contributes to a wide range of pathological processes that include impaired growth and development, increased oxidative stress, elevated inflammatory signaling, and apoptosis induction [24–27]. Zinc deficiency exacerbates negative health outcomes in individuals with chronic diseases, such as diabetes, chronic liver disease, Alzheimer's, and cardiovascular diseases [28–31]. Mutations in certain zinc transporters have been linked to intellectual disabilities, developmental delays and short stature [32], and skeletal dysplasia, among other disorders [33]. Specifically, mutations in the *Znt2* transporter in breastfeeding mothers are associated with neonatal ZD due to low milk zinc concentration [34]. Alternatively, zinc excess, while rare, is also detrimental with symptoms that include nausea and headaches during acute zinc toxicity to reduced immune function and neuropathy in chronic zinc excess [21].

The essential need for zinc is particularly notable within the mammalian reproductive system where ZD causes abnormal or failed development of germ cells in both sexes, which can result in infertility. In fact, approximately 9% of couples [35] face issues with infertility where dietary zinc status and/or dysregulation of the metal may play a significant role. Zinc deficiency during pregnancy increases the risk of adverse outcomes that include miscarriage, fetal growth retardation, impaired neural development, and placental dysfunction, supporting the recommendations for pregnant and breastfeeding women to increase their zinc intake [19, 36–39].

Over the past decade, work from many labs have uncovered many zinc-dependent processes that regulate mammalian female reproduction (Table 1). These discoveries were made using various techniques for the visualization and quantification of zinc ions in cells (Table 2). However, many questions remain unanswered. Revealing the roles of zinc in female reproduction, in addition to the reproductive defects and impaired fertility caused by ZD, is therefore critical for the identification, treatment, and prevention of infertility and ZD-associated risks to prenatal and postnatal health. The aim of this review is to highlight recent advancements in our understanding of zinc-mediated reproductive processes in mammalian female reproduction and the pathologies and phenotypes caused by ZD in reproductive health.

Zinc control of ovarian function

Primordial follicles form during fetal development or shortly after birth in mammals when oogonia, arrested at the diplotene stage of prophase I, become surrounded by somatic cells [40–45]. Interestingly, the somatic cells destined to enclose oogonia and become granulosa cells (GCs) can arise from either the coelomic or the ovarian surface epithelium [44, 45]. Primordial follicles remain in a quiescent and dormant state until they are "activated" to begin follicle growth. Follicle activation involves a complex interaction between germ cells and somatic factors to ensure steady maintenance of the growing follicle pool [46, 47]. Little information is available on the importance of zinc in these early stages, however, recent work in nematodes suggests a necessary role for zinc in regulating early meiosis [48] (see discussion below).

Following activation, gonadotropin-independent growth of the primary oocyte and proliferation of the somatic GCs contribute to the follicle development to the preantral stage [49, 50]. Shortly before the preantral to antral transition, growth of preantral follicles becomes dependent on circulating gonadotropins, particularly follicle stimulating hormone (FSH). Gonadotropin support is essential to stimulate further growth of antral follicles to the preovulatory stage. The GCs also develop along two distinct lineages during antrum formation; the cumulus GCs (CGCs), which immediately surround the oocyte, and the mural GCs (MGCs) that line the follicular wall [51–54]. Paracrine signaling among theca cells, GCs, and the oocyte regulates many processes in the follicle, including proliferation, steroidogenesis, metabolism, meiosis, and ovulation [53–75]. As with the early germ cell development, there is little information on the role of zinc in the follicle assembly, activation, or preantral follicular growth. However, there have been several recent studies showing effects of in vivo or in vitro zinc depletion on the development of antral follicles and function of the cumulus–oocyte complex (COC). These are reviewed in the following sections.

Zinc in early germ cell meiosis

There is no information to date on the role of zinc in early follicular or germ cell development in mammals. However, two recent studies suggest an important role for zinc ions during both early and late germ cell developments in nematodes. Most notably, *Caenorhabditis elegans* grown under zinc-limiting conditions using the chelator N,N,N′,N′-tetrakis(2-pyridylmethyl)ethane-1,2-diamine (TPEN) show a severe reduction in fertility, which is mostly due to the impaired oocyte development rather than the deficiencies in sperm production [48, 76]. *Caenorhabditis elegans* are hermaphrodites

Table 1. Studies showing the importance of zinc according to species and stage of development.

Species	Oocyte development, maturation, and ovulation			Fertilization and partheno-genesis		Preconception and periconception effects		Reference(s)
	In vitro		In vivo	In vitro	In vivo	Early embryo	Fetal and placenta development	
	DO	COC						
Mouse	X							[127]
	X	X	X					[56, 87]
	X			X				[10, 104, 117, 121, 124, 125, 132]
	X					X		[154]
		X						[118]
		X	X	X		X		[98]
		X	X	X	X	X		[6]
				X				[11, 233]
				X		X		[141]
						X		[163, 164, 202]
						X	X	[88]
							X	[210, 216, 226]
Rat							X	[197]
Primate	X			X				[10]
Human				X				[11]
Bovine		X						[151, 152]
		X		X				[123]
		X		X		X		[150]
Porcine		X						[103, 234]
		X				X		[235]
		X		X		X		[143, 155]

and progress through four larval stages (L1–L4) before reaching adulthood [77, 78]. The gonad is composed of two U-shaped gonads, each composed of a distal arm, gonadal bend, proximal arm, and ending in a common uterus [79]. In the distal gonad, a stem cell population supplies germ cells for spermatogenesis during the L3 larval stage but switches to oogenesis beginning around L4 [80, 81]. During oogenesis, there is incomplete cell division in the distal gonad, resulting in the formation of a syncytium called the rachis. Germ cells enter meiosis and progress to the pachytene stage of prophase I while joined with other germ cells in the rachis. As cells enter the proximal gonad, they progress through the final stages of meiosis and become enclosed by a membrane to produce an orderly arrangement of fully grown oocytes which will ovulate in succession.

Oocyte development in worms normally progresses through diplotene and eventually diakinesis in the proximal gonadal arm [79]. However, in TPEN-treated worms, there is a notable extension of pachytene germ cells well into the proximal gonadal arm [48]. These data may indicate that zinc is essential for early meiotic progression; however, details on the cellular mechanisms affected by zinc depletion remain to be determined. The early germ cell arrest could be the result of disruption in the mitogen-activated protein kinase (MAPK) signaling pathway, which promotes meiotic progression in worms and is known to be regulated by zinc in other contexts [82, 83]. Ablation of the MAPK homolog mitogen-activated protein kinase 1 (MPK-1) results in pachytene arrest in *C. elegans* [84, 85]. The MPK-1 interacts with the zinc-containing protein and mutants for both gla-3 and show a similar gonadal phenotype to the TPEN-treated worms [86]. It will be interesting to examine

the details of zinc involvement in early meiosis in mammals. In late meiosis, around ovulation, there are many similarities between worms and mammals, suggesting a robust conserved pathway. For example, as in mice (see below), there are defects in the polar body extrusion and spindle formation, ultimately resulting in aneuploidy, in the TPEN-treated worms [76].

Zinc in antral follicular development

Antral follicles are actively growing, highly steroidogenic structures that are regulated by autocrine, endocrine, and paracrine signals during growth and ovulation. Recently, a series of studies using dietary zinc depletion during antral follicular development and ovulation revealed an important role for zinc during follicle rupture and cumulus expansion [6, 87, 88]. In these studies, newly weaned mice ~18 days of age were fed a control (>30 mg zinc/kg) or zinc-deficient (<1 mg/kg) diet for up to 10 days. The timing of the dietary treatments was important because, at weaning, there is a cohort of large preantral follicles that are poised to make the preantral to antral transition and form a synchronous follicular wave. These follicles will ovulate if stimulated with exogenous hormones. Using this paradigm, the authors show that neither a 3- nor a 5-day treatment with a ZD diet causes any decrease in the ovulation rate in response to exogenous hormonal stimulation [6, 87]. However, a 10-day treatment with a ZD diet resulted in an almost complete ovulation block. Interestingly, the block in follicle rupture was not due to the failure of follicular development. In fact, antral follicles grew quite large in the ZD group, but did not rupture, even after hormonal stimulation with an ovulatory dose of human

Table 2. Examples of commonly used tools and methods to study zinc.

Type	Method	General description
Manipulation of zinc availability	Membrane-impermeable chelators	Compounds with high zinc affinity that are unable to cross the lipid bilayer of cells to manipulate zinc availability in media. One example is zinc-diethylenetriamine pentaacetate (Zn-DTPA) that has a high affinity for zinc but also forms complexes with other heavy metals such as actinides
	Membrane-permeable chelators	Compounds with zinc affinity that cross the plasma membrane to enter cells to sequester intracellular zinc. The most commonly used in reproductive biology is TPEN N,N,N′,N′-tetrakis(2-pyridylmethyl)ethane-1,2-diamine, which has a high affinity for zinc but also binds other transition metal ions such as copper and iron
	Zinc ionophore	Increases intracellular zinc uptake. A potent zinc ionophore is zinc pyrithione, a derivative of pyrithione that is also known to mediate influx of other ions such as copper, is used in vitro
	Dietary ZD	Used in animal models to assess in vivo effects of ZD. Zinc-deficient diets typically consist of <3 mg zinc/kg in the diet compared to control diets of >= 29 mg zinc/kg
Fluorescence probing (reviewed in [236, 237]	Small molecule indicators	Zinc-selective fluorophores that range in sensitivity, typically have cell permeable and impermeable versions, and are easy to use in cell culture. However, these probes typically have similar excitation and emission spectra, and assumptions that these indicators only bind labile zinc appear to be invalid. Examples used commonly in reproductive biology include FluoZin-3 and Zinquin
	Peptide-based zinc indicators	Exploits naturally derived zinc-binding peptides with fluorescent sensors. These probes have a greater range of excitation and emission wavelengths and can target specific subcellular compartments; however, they are more difficult to use and have a smaller dynamic range compared to small molecule indicators. Includes single protein-based zinc fluorophores, Forster resonance energy transfer (FRET)- and bioluminescence resonance energy transfer (BRET)-based probes. Examples include ZnGreen, Zap, and BLZinCh
Total elemental imaging and mapping (reviewed in [236])	X-ray fluorescence microscopy	High-resolution technique that can image total (free and bound) zinc localization and quantify zinc abundance by the atom. Includes synchrotron X-ray fluorescence, X-ray absorption spectroscopy, and energy-dispersive spectroscopy
	Mass-spectrometry-based imaging	Employs ionization methods to detect particles by their mass-to-charge ratio. Examples include laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) and secondary ion mass spectrometry (SIMS)

chorionic gonadotropin [87]. Impaired follicle rupture could be due to the decreased activity of zinc-dependent enzymes, such as matrix metalloproteinases, which are important for the degradation of the follicular wall during ovulation [89–92]. However, effects on other cellular proteins or pathways cannot be ruled out.

Another zinc-dependent pathway has recently been discovered in the ovarian cells of the Atlantic croaker and zebrafish [93, 94]. In these species, testosterone-mediated zinc signaling regulates cell survival (croaker) and oocyte maturation (zebrafish). In GCs, mammary cancer, and prostate cells of these model species, androgens signal through the activation of a previously identified zinc transport protein, Zip9 [93, 95, 96]. These studies provide compelling evidence that Zip9 functions as both a zinc transporter and membrane androgen receptor coupled to both zinc transport and G-protein activation. While, to date, no studies have identified a role for Zip9 in mammalian ovaries, androgen-mediated signaling through Zip9 could represent another zinc-mediated pathway that is important for

Table 3. Studies that highlight the evolutionarily conserved zinc efflux upon egg activation.

Species	Reference(s)
Mouse	[10, 121, 125, 141, 146, 233, 238]
Bovine	[123]
Human	[11, 238]
Nonhuman primate	[10]
Fly	[239]
Zebrafish	[97]
Frog	[240]

the ovarian function in mammals, particularly since Zip9 in zebrafish is required to generate a zinc spark, a phenomenon that is conserved across many species (Table 3) [97].

Zinc in the COC

In antral follicles, the COC is particularly sensitive to zinc depriva-tion. The effects of zinc depletion on oocyte function are discussed below. Here, we consider how zinc ions may contribute to the function of the cumulus cells. In unstimulated COCs, chelation of transition metals, including zinc, with TPEN causes an acute increase in steroidogenic transcripts, such as *Cyp11a1* and *Star* mRNA, and an increase in progesterone accumulation in the culture medium [87, 98]. This is likely due to zinc depletion because feeding a zinc-deficient diet for 10 days recapitulates the increase in steroidogenic transcripts in COCs [87]. Interestingly, luteal tissue cultured ex vivo also responds to TPEN chelation with an increase in the abundance of steroidogenic transcripts and progesterone accumula-tion in culture media [98]. Collectively, these observations suggest that zinc may play an inhibitory role in the onset and mainte-nance of progesterone production. However, this idea remains to be tested rigorously. The increase in progesterone under zinc-depleted conditions could also be due to the removal of inhibitory path-ways. Indeed, signaling through the zinc-binding SMAD (Mothers against decapentaplegic homolog) transcriptional pathway is known to inhibit progesterone production [99–101], and TPEN treatment potently suppresses SMAD2/3 phosphorylation in COCs [87]. The suppression of SMAD2/3 signaling also leads to other defects includ-ing a complete failure of cumulus expansion as seen in the TPEN-treated COCs or COCs from animals fed with a zinc-deficient diet [87]. Further, CGCs isolated from mouse COCs cultured in TPEN show a dramatic increase in the expression of *Cyp11a1* and *Lhcgr* typically associated with the MGCs [87]. While phosphorylation of SMAD2, which suppresses mural transcripts and enables cumulus expansion following epidermal growth factor (EGF) stimulation, is also markedly reduced in CGCs by zinc chelation, EGF-induced mitogen-activated protein kinase 3/1 (MAPK3/1) phosphorylation does not appear to be disrupted [51, 87].

Together, these findings indicate that zinc has a role in main-taining the cumulus phenotype and may be involved in preventing the premature upregulation of progesterone production in the COC [98]. Thus, a model is emerging wherein zinc ions are required for the SMAD activation to prevent the premature upregulation of proges-terone and to enable cumulus expansion. The SMAD2/3 activation is reduced by 6–8 h following the onset of cumulus expansion [51], which may be a similar timeframe for a shift in free intracellular zinc from the CGCs to the oocyte [56]. It is tempting to speculate that the release of zinc from the CGCs may play a functional role as a switch in the CGC physiology at the time of ovulation. This idea requires further experimental study and pathways other than SMAD activation may be involved. Nevertheless, it is interesting that CGCs, which have extensive gap junctional communication with the oocyte [102], have much higher free intracellular zinc stores than the oocyte before maturation [56].

It seems that the CGCs themselves produce a factor (zinc inhibitory factor (ZIF)) to regulate the level of free intracellular zinc in the oocyte before ovulation. Removal of CGCs leads to an abrupt increase in the oocyte free intracellular zinc which is reversed after co-culture with CGCs. Thus, ZIF is a secreted product that reduces free intracellular zinc in the oocyte [56]. Treatment with EGF abolishes the ZIF activity, allowing free intracellular zinc in the oocyte to increase [56, 103, 104]. However, metaphase II (MII) oocytes remain sensitive to ZIF since the CGC co-culture reduces free intracellular zinc in these cells. Whether the zinc that accumulates in the in vivo maturing oocyte originates from the extracellular milieu,

through a paracrine mechanism that involves flux of labile zinc from the CGCs to the oocyte, or a combination thereof and to what degree, is unknown [56].

Zinc regulation of oocyte development and maturation

Prophase I is maintained in the germinal vesicle (GV) oocytes through an orchestrated mechanism where MGCs synthesize natri-uretic peptide precursor C (NPPC) that signals through its receptor, NPPC receptor 2 (NPR2), in CGCs to produce cyclic guanosine monophosphate (cGMP) production [67, 105]. The cGMP diffuses to the oocyte via the connexin 37 (Cx37)-predominant gap junctions to inhibit phosphodiesterase 3A (PDE3A), thus blocking cyclic adenosine monophosphate (cAMP) breakdown and inhibiting the activation of maturation induced downstream by maturation promoting factor (MPF) [64, 67, 105]. Ovulatory signals abolish this inhibitory pathway, leading to a decrease in cAMP, MPF activation, and subsequent GV breakdown (GVBD) and progression to MII (Figure 1).

Sufficient free intracellular zinc levels are required to maintain prophase I arrest in GV-stage oocytes, but once maturation is initi-ated, a rise in intracellular zinc is necessary for the oocyte to progress through meiosis I, extrude an asymmetric polar body, establish a second meiotic spindle, and arrest at MII. Consequently, insufficient zinc availability severely disrupts oocyte maturation, which results in a diverse set of phenotypic abnormalities depending on the severity, duration, and maturation stage in which the ZD occurs. These disordered morphologies include the formation of a large polar body, symmetric cell division, reduced cumulus expansion, and meiotic failure before MII. All of these defects result in a failure of maturation or subsequent fertilization and embryonic development. This section will outline the physiological functions of intracellular zinc during oocyte maturation and how misregulation of the transition metal may lead to developmental failure.

Follicular signaling pathways in ovulation

Depending on the species, one or multiple preovulatory follicles are induced to ovulate by a surge of circulating luteinizing hormone (LH) released by the anterior pituitary. The LH receptors (LHRs) in MGCs respond to the LH surge by increasing cytosolic cAMP to activate protein kinase A (PKA), resulting in the release of the EGF-like peptides (EGF-LPs) amphiregulin (AREG), epiregulin (EREG), and betacellulin (BTC), which have autocrine and paracrine functions [65, 66, 106]. These EGF-LPs, particularly AREG and EREG, bind to the EGF receptors present in CGCs, which generally lack LHRs in mice, to reduce the cGMP production and terminate Cx37-predominant gap junctions between the CGCs and the GV oocyte [51, 66, 106, 107]. Both effects result in a reduction of intra-oocyte cGMP, allowing an increase of PDE3A, and resulting in the degradation of cAMP which was high in GV oocyte through constitutively activity of G-protein coupled receptor 3 (GPR3) and G-protein coupled receptor 12 (GPR12) [64, 105, 108–111]. The cAMP degradation inactivates PKA and thus facilitates the activa-tion of MPF, a protein complex consisting of an enzymatic cyclin-dependent kinase (CDK1) and PKA-regulated cyclin B1 (CCNB1) [112]. The MPF activation occurs through both the reduction of WEE1B activity that phosphorylates and deactivates CDK1 at Tyr15 and the inhibition of the anaphase-promoting complex/cyclosome
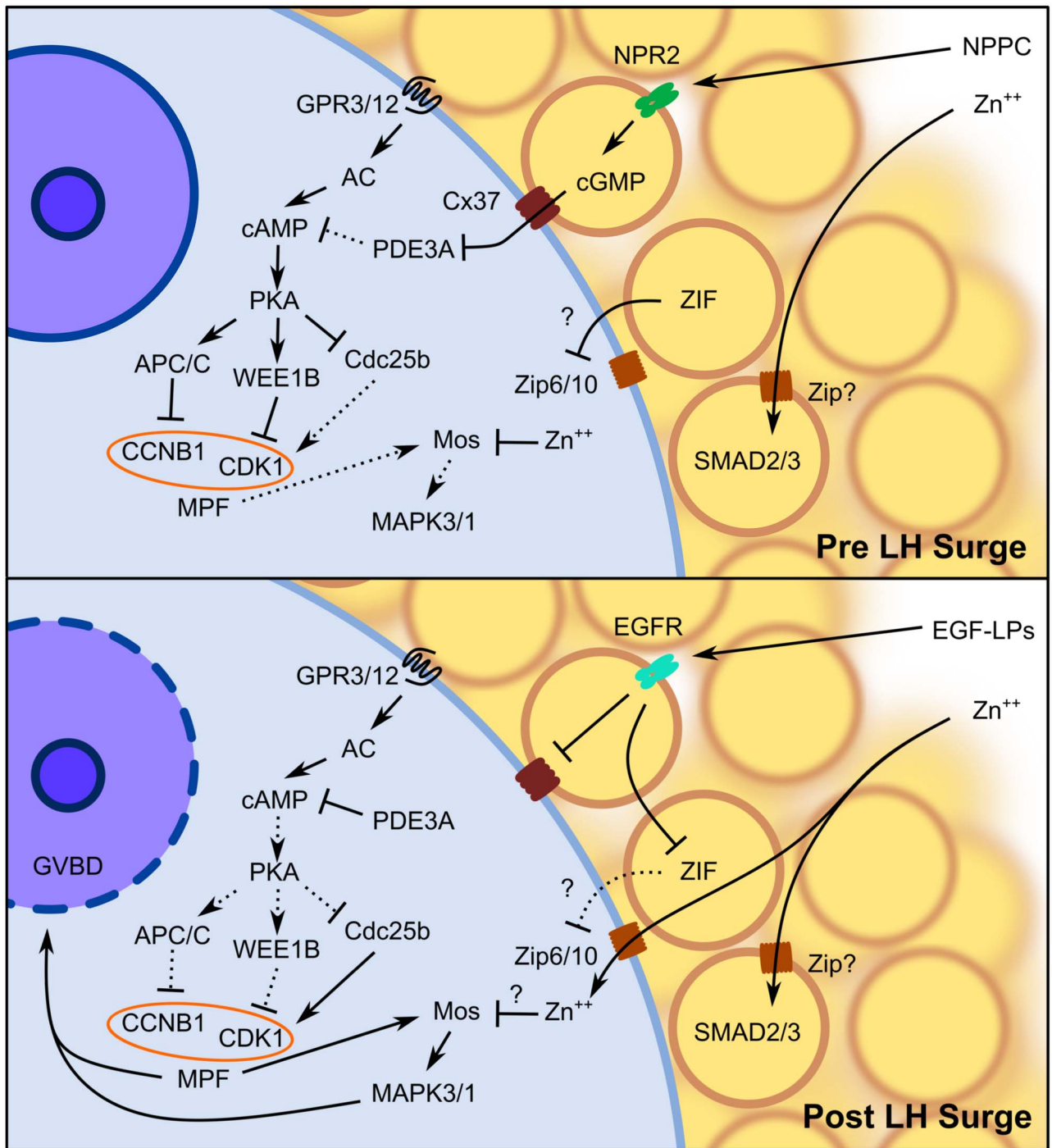
Figure 1. Diagram of the signaling pathways that regulate GV arrest and GVBD. The NPPC secreted from peripheral MGCs binds to NPR2 in CGCs to upregulate cGMP synthesis. The cGMP passively diffuses into the GV oocyte via Cx37 predominant gap junctions to inhibit PDE3A, thus maintaining an elevated concentration of cAMP in the oocyte through constitutive activation of AC by GPR3/12. The high concentration of cAMP activates PKA to directly inhibit MPF through APC/C proteasomal degradation of CCNB1 and WEE1B phosphorylation of CDK1 in addition to inhibiting Cdc25b activity. The CGCs also secrete ZIF that prevents uptake of free intracellular zinc by the oocyte, possibly through inhibition of Zip6/10. In the CGCs, zinc is required for SMAD2/3 activation to maintain the cumulus phenotype, while in GV oocytes, zinc inhibits premature activation of the Mos–MAPK3/1 pathway. Following the LH surge, EGF-LPs secreted by MGCs bind to the EGFR in CGCs to initiate the closure of CGC–oocyte gap junctions, thus preventing further cGMP diffusion into the oocyte. Activation of the EGFR in CGCs also inhibits ZIF activity, allowing free intracellular zinc accumulation in the oocyte during maturation. The reduction in intra-oocyte cGMP concentration increases PDE3A activity to degrade cAMP. As PKA activity is subsequently reduced, MPF is activated by increased CCNB1 availability and CDK1 dephosphorylation by Cdc25b, leading to both activation of Mos–MAPK3/1 and GVBD. Abbreviations: AC, adenylyl cyclase; EGFR, epidermal growth factor receptor; Zn^{++}, zinc. Arrows indicate activation and bars represent inhibition. Solid lines indicate active pathways, while dashed lines represent inactive pathways.

(APC/C) proteasome to reduce CCNB1 degradation. The active MPF protein complex induces GVBD and meiotic resumption [64, 67, 105, 108, 109, 113]. Additionally, EGF receptor activation and gap junction closure initiate CGC expansion and mucification [64, 67, 105].

Following the LH surge, the mammalian oocyte will undergo a period of meiotic maturation for approximately 12–16 h until arresting at MII, where it will await possible activation at fertilization. During this short window, the oocyte will undergo a wholesale reorganization of the cytoplasm [114] as well as remodeling of the transcriptome and proteome [115, 116] to prepare for early embryonic development. Proper maturation to the MII stage is critical in producing a high-quality oocyte, which largely determines the fertilization success and the development of healthy embryos and progeny.

Intracellular zinc maintains GV arrest

Interestingly, in vitro zinc sequestration by TPEN alone induces GVBD in GV oocytes arrested with a PDE inhibitor, suggesting that sufficient zinc is necessary for maintaining prophase I arrest [87, 117]. The GV-stage oocytes maintained in media containing TPEN undergo GVBD and subsequently arrest prematurely at telophase I. However, oocytes that undergo only a transient TPEN exposure to induce GVBD and then are cultured without the chelator and sufficient zinc can successfully mature to the MII stage, while maintaining normal developmental competency to blastocysts [87, 117]. The role of zinc in meiotic prophase I arrest appears to be independent of the physiological mechanisms that regulate maturation as TPEN-induced GVBD occurs even in the presence of various PDE inhibitors and without a reduction in the intra-oocyte cAMP concentrations [87]. Instead, GVBD induced by zinc chelation occurs through the activation of the Mos–MAPK pathway [117, 118].

In mouse oocytes, Mos is only expressed after the induction of maturation and acts to maintain MII arrest by maintaining MPF prior to fertilization, however, injection of Mos can induce GVBD [119, 120]. The TPEN-induced maturation in prophase I-arrested oocytes appears to be through premature activation of Mos–MAPK, which precedes GVBD and MPF activation, while cAMP, which maintains prophase I arrest, increases [87, 117, 118]. Surprisingly, while TPEN-induced GVBD is regulated by the expression of Mos, the failure to transition from meiosis I to meiosis II during ZD is unrelated to the Mos–MAPK pathway even though similar phenotypes are observed in Mos insufficiency [118]. Similar abnormalities have been observed both in vivo using a ZD diet and in vitro using zinc chelators, where early GVBD occurs without an ovulatory signal and results in the premature meiotic arrest that impairs both ovulation and oocyte transport to the oviduct [87].

Accumulation and localization of zinc during maturation

Some of the first studies that identified the necessary role of zinc in mammalian reproduction observed the importance of the transition metal for oocyte maturation. During this short developmental window, the total intracellular zinc content increases by 30–50%, an approximate influx of 20 billion atoms [104, 121]. However, the amount of zinc accumulated during maturation is likely greater as these studies employed denuded oocytes for which the removal of CGCs prompts an immediate influx of zinc in the GV-arrested oocytes to approximately 2-fold after 15 min and to 4-fold after 1 h [56]. The accumulated zinc is predominantly stored in vesicles that are located symmetrically along the oocyte cortex at the GV stage and polarize toward the vegetal pole at the MII stage [10, 121–124]. Intriguingly, these zinc-positive vesicles relocalize to and from the cortical region in a meiotic-stage dependent manner, forming a hemispherical pattern by the MII stage with zinc-positive vesicles localized away from the spindle [121, 125].

Zinc in meiotic progression and spindle formation

The transporters Zrt- and Irt-like protein 6 (Zip6) and Zrt- and Irt-like protein 6 (Zip10) both belong to the Slc39a family that regulates zinc influx into the cytoplasm, and both localize to the oocyte cortex [122]. These two transporters are necessary for the oocyte maturation in mice as the functional blocking of the proteins with anti-Zip6 and anti-Zip10 antibodies or morpholino-induced knockdown of translational capacity for either transporter disrupt this process. The mechanism of disrupted oocyte maturation likely involves interference of Zip6–Zip10 heteromer formation observed in zebrafish [126], which produces phenotypes that resemble zinc-chelated oocytes [122]. Restricting intracellular zinc with the chelator TPEN during mouse in vitro oocyte maturation results in the meiotic arrest at telophase I, formation of an abnormal spindle, and in some cases, formation of an abnormally large polar body or even symmetric cellular division [104, 118]. While telophase I-arrested mouse oocytes are fertilization-competent as pronuclear formation still occurs, embryonic progression to the blastocyst is impaired [104].

A sufficient intracellular zinc supply is critical immediately prior to polar body extrusion and meiotic I exit. Zinc supplementation of maturing oocytes cultured in the zinc chelator TPEN rescues MII morphology when provided prior to telophase I arrest but not after [104]. Additionally, TPEN-mediated zinc chelation during the MI to MII transition, at approximately 7.5 h after GVBD, prevents MII spindle formation [127]. The failure of zinc-insufficient oocytes to transition from meiosis I to meiosis II is due to impaired MPF activity [118]. The CCNB1, a component of MPF, through its translation and degradation, is one control point for MPF activity during the MI to MII transition [118, 127, 128]. The MPF activity is increased in the oocyte to induce GVBD, is reduced during meiosis I and extrusion of the first polar body, and then is again upregulated to establish the MII spindle [129]. However, zinc-insufficient oocytes fail to accumulate CCNB1 and upregulate the MPF activity following meiosis I. As a result, they fail to enter MII and remain in telophase I arrest [118, 127]. Early mitotic inhibitor 2 (EMI2), a zinc-binding APC/C proteasome inhibitor, is an integral component of the cytostatic factor (CSF) to initiate MII entry and maintains MII arrest which is disrupted in zinc-insufficient maturing oocytes [127, 130–132]. Reduced zinc binding to EMI2 leads to an overactive APC/C proteasome which increases CCNB1 degradation and reduces MPF activity, resulting in early meiotic arrest [127].

Zinc in actin nucleation and meiotic spindle migration

Abnormal meiotic progression and large polar body extrusion resulting from zinc chelation appears to be caused by the disruption of actin cytoskeletal nucleation and thus spindle formation and

localization to the cortex. Culture in TPEN results in reduced F-actin in a dose-dependent manner, and disruption of Zip6 and Zip10 by morpholinos significantly reduces actin as measured by phalloidin intensity [124]. A highly specialized actin cytoskeleton facilitates spindle formation and positioning as oocytes from many species lack centrosomes typically used to facilitate mitotic division. The lack of centrosomes may be beneficial during polar body extrusion by allowing the meiotic spindle to localize more closely to the cortex and by reducing the cytoplasmic loss during division [133–135]. This actin cytoskeleton is nucleated through interactions between Formin-2 and Spire1/2 which localize to Rab11a-positive vesicles, which serve as the site for nucleation and drive asymmetric division [135–137]. Importantly, Spire1/2 contains a cysteine-rich zinc finger domain that is crucial for its localization, and zinc content regulates Formin-2/Spire1/2 localization and actin nucleation [124, 138]. Further, Spire1/2 co-localizes with punctate zinc-positive vesicles within the oocyte [124].

Spire1/2 knockdown or mutation of the zinc-binding domain results in reduced cytoplasmic actin mesh formation, premature meiotic arrest, and abnormal polar body extrusion similar to zinc chelation by TPEN [104, 124, 137]. Both zinc chelation and specific mutation of the Spire1/2 zinc finger disrupts its localization, thus dispersing the nucleator throughout the cytoplasm and reducing contact with Formin-2 [124]. While zinc chelation results in a loss in MPF activity after the first meiotic division, impaired actin mesh formation by zinc chelation is not recovered by supplementing MPF activity with a non-degradable form of CCNB1 [118, 124]. However, this could be due to zinc acting through cell division cycle 25 (Cdc25) to regulate MPF through CDK1, thus supplementation of the other component of MPF may not be sufficient to rescue the MPF activity [139]. Importantly, Spire1/2 contains a zinc finger motif and co-localizes with zinc to cortical granules (CGs) along the oocyte membrane, where zinc chelation and a Spire mutant lacking the zinc-binding domain both impair Spire localization [121, 124, 136, 137].

Together, these studies have identified zinc as a critical mediator throughout the oocyte maturation process to maintain prophase I arrest, regulate the meiosis I to meiosis II transition, and initiate MII arrest.

Zinc in fertilization and egg activation

Mature eggs arrest at MII of meiosis and await fertilization and egg activation (Figure 2). The MII arrest is maintained by a CSF which we now know is dependent on the accumulation of enough free intracellular zinc during maturation. As described earlier, accumulated zinc in maturing oocytes binds to and activates EMI2 which disrupts the association of cell division cycle 20 (Cdc20) with the APC/C proteasome complex and thereby inhibits the degradation of CCNB1 to maintain MPF-mediated meiotic arrest [127, 130–132]. Upon fusion of the sperm and egg, phosphoinositide phospholipase C γ1 (PLCγ1) is delivered from the sperm to the oocyte to induce the conversion of inositol trisphosphate (IP3) and diacylglycerol from phosphatidylinositol 4,5-bisphosphate at the oocyte membrane, thus stimulating calcium release from the intracellular stores and activating protein kinase C, respectively [123, 140]. The resulting transient calcium oscillations induce zinc sparks, which are short bursts of zinc released into the extracellular milieu that result in a reduction of the total zinc status of the fertilized egg [10, 11, 121, 123, 141].

One to five zinc spark events are triggered within 90 min of fertilization or parthenogenesis in mammals, and approximately half of the 20 billion zinc atoms accrued during oocyte maturation are released prior to the cleavage to form the two-cell embryo [10, 11, 121, 123, 141]. While multiple zinc sparks may be initiated in a single cell, each preceded by an intracellular calcium oscillation, only one is required for the successful resumption of meiosis II and transition from an egg into an embryo [10, 11, 121, 123, 141]. Instead, it is the amplitude of the zinc released during these events that is critical as a sufficient drop in intracellular zinc is necessary for meiotic resumption [10]. The amount of zinc released positively correlates with the chance that the resulting embryo will develop into a morula and blastocyst [141]. Additionally, the zinc spark event is stage-dependent as GV oocytes respond to the parthenogenetic activation with a much smaller amplitude relative to MII oocytes [11].

The quick release of zinc leads to a reduction in the total intracellular zinc available to bind to and activate EMI2, thus relieving the inhibition on the APC/C proteasome to increase CCNB1 degradation and alleviate MPF-mediated meiotic arrest [10, 121, 127, 130, 142]. The calcium oscillations stimulated by IP3 work synergistically to resume meiosis II by signaling for the degradation of EMI2, further reducing APC/C proteasomal inhibition [127, 130]. Calcium release is not required for in vitro meiotic resumption in MII oocytes as zinc chelation by TPEN induces MII exit through the APC/C proteasome pathway to result in parthenogenesis without calcium mobilization [130, 143]. However, zinc chelation-induced parthenogenesis causes significant reductions in developmental potential, with successfully activated oocytes rarely progressing beyond the two-cell embryonic stage [130]. In separate studies with pig oocytes, brief TPEN exposure for 30–60 min promoted, while longer exposures inhibited the blastocyst development [143, 144]. Conversely, zinc excess also is detrimental to developmental potential as displayed in experiments with the ionophore zinc pyrithione that increased cortical actin and blocked pronuclear formation in eggs which had successfully completed zinc spark events prior to treatment [10].

Extra-oocyte role of zinc sparks

In addition to inducing MII exit upon successful fertilization, the zinc spark events also act to prevent polyspermy. Approximately, 500–600 million atoms or 5% of the total zinc released following egg activation is incorporated into the surrounding ZP without affecting the exocytosis of CGs or ZP protein cleavage [125]. The accumulation of zinc along the ZP increases fibril connectivity along the glycoprotein matrix, which reduces the number of sperms that are capable to bind and reach the recently fertilized egg [125, 145, 146]. Additionally, zinc released into the extracellular milieu reduces the local sperms' forward motility to further reduce the number of sperms reaching the egg [146]. This was a particularly interesting finding as hardening of the ZP to prevent additional sperm binding does not occur until around 30 min after fertilization, while the zinc spark events are initiated only a few minutes after fertilization, indicating that early zinc efflux is used to slow sperm to allow more time for cleavage and inhibition of sperm-binding proteins along the outer zona matrix and to completely block polyspermy [146].

Epigenetic defects in fertilized zinc-deficient oocytes

The research discussed above outlines some of the mechanisms of zinc-mediated processes during maturation and fertilization using in vitro methods; however, there is ample evidence that zinc depletion in vivo impairs the female reproductive function. An acute bout of ZD prior to ovulation reduces the in vitro fertilization potential
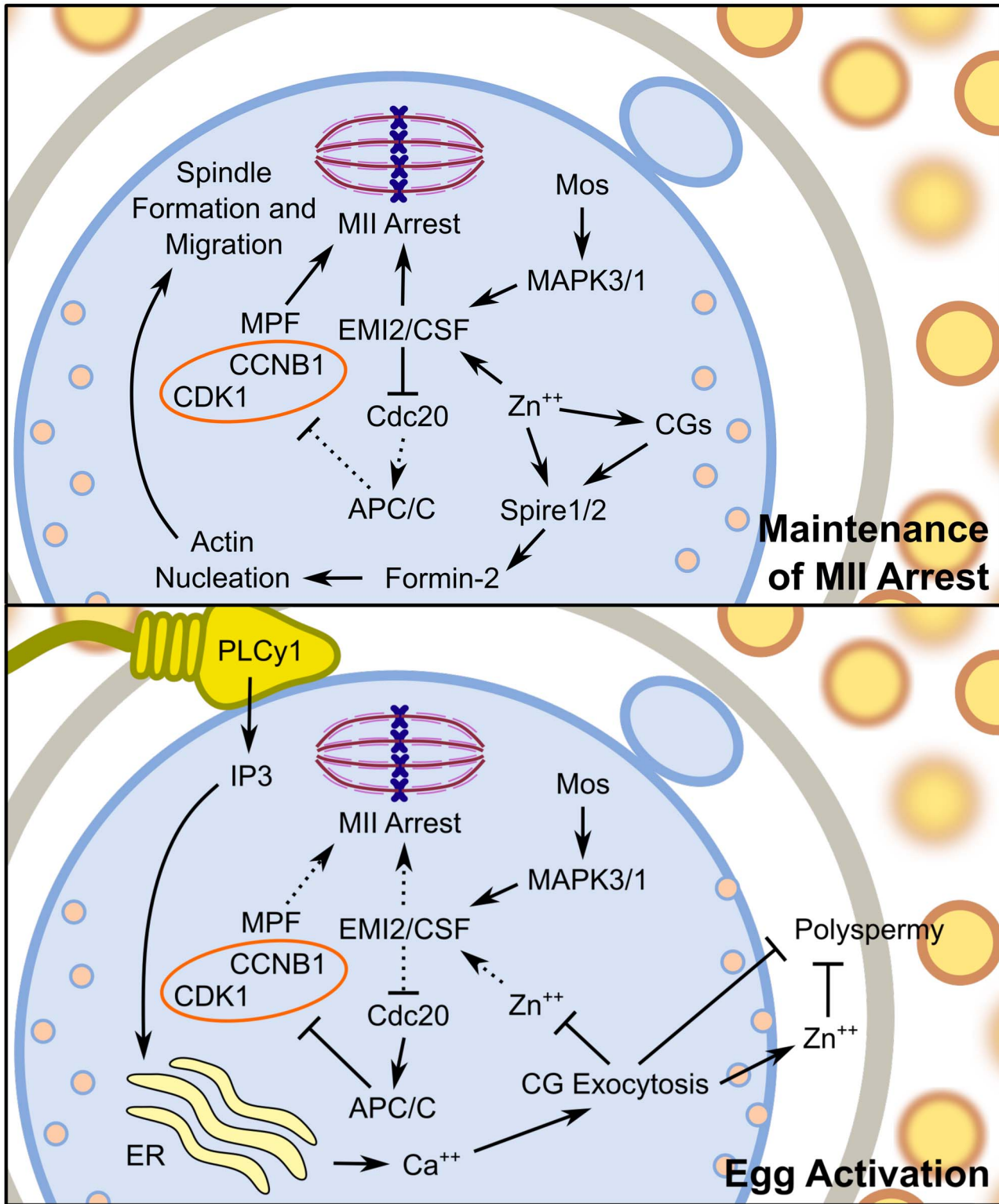
Figure 2. Model of signaling pathways that modulate MII arrest and egg activation. A high intracellular concentration of zinc establishes and maintains MII arrest through activation of EMI2 to prevent degradation of CCNB1 by the APC/C proteasome and maintains high MPF activity to maintain MII arrest. Additionally, zinc accumulation facilitates Spire1/2 localization with Formin-2 to nucleate actin cytoskeleton and to facilitate meiotic spindle formation and migration to the cortex. Following sperm binding and the acrosomal reaction, PLCy1 increases IP3 availability that initiates calcium release from the ER. This calcium influx promotes exocytosis of CGs, releasing zinc and other factors important for establishing the block to polyspermy. The subsequent reduced intracellular concentration of zinc reduces EMI2 activity. Subsequent activation of the APC/C proteasome by Cdc20 leads to the degradation of CCNB1 to reduce MPF activity, thus allowing completion of meiosis. Abbreviations: Ca^{++}, calcium; ER, endoplasmic reticulum; Zn^{++}, zinc. Arrows indicate activation and bars represent inhibition. Solid lines indicate active pathways, while dashed lines represent inactive pathways.

even when the ovulation rates are unaffected [6]. Although in vivo fertilization rates are not as dramatically reduced in ZD mice compared to in vitro studies, the negative effects of ZD are present in the fertilized eggs from these animals, which displayed a disorganized chromatin structure and the resulting embryos had reduced developmental competency compared with controls [6]. As discussed below, ZD impairs histone (H3K4me3) and DNA methylation. Indeed, deletion of mixed-lineage leukemia 2 (Mll2), also known as lysine methyltransferase 2D (Kmt2d), the methyltransferase responsible for H3K4me3, results in maturation failure and oocyte death [147]

Supplementation with the methyl donor S-adenosylmethionine (SAM) during in vitro maturation restores H3K4me3 and improves the proportion of ZD oocytes that are successfully fertilized and progress to the two-cell embryo stage, indicating that disrupted histone methylation is a hallmark of disrupted fertilization and early embryonic development caused by ZD [6]. While sperm may enter the oocyte and decondense to initiate some of the early stages of fertilization in zinc-insufficient oocytes, they fail to form blastocysts as a result of either a failure to reach MII or pronuclear fusion [6, 68, 141]. In vivo, preconception ZD in mice reduces fertilization success by 20% after 3 days, but fertilization rate is reduced by over 90% after 5 days of ZD, nearly as effective as in vitro zinc chelation [88]. Since biological zinc reserves are low, animals are likely able to maintain a sufficient supply of zinc for oocyte maturation for only a few days under ZD conditions. Despite the similarities of ZD in vivo and chelation in vitro on oocyte maturation and fertilization, the exact mechanisms altered by ZD in vivo remain to be fully identified but do include alterations in the epigenetic programing of the oocyte and perhaps zygote as discussed below. Overall, it is likely that zinc concentrations fall below the threshold necessary for successful oocyte maturation and pregnancy.

Zinc supplementation in IVM and IVF

Prior to uncovering the role of zinc in regulating mammalian oocyte maturation and fertilization, experiments with COCs supplemented with zinc during IVM showed a positive correlation between zinc concentration and the rate of blastocyst formation following IVF [148]. Increased intra-oocyte, but not intra-CGC, glutathione (GSH) concentrations and reduced DNA damage in CGCs are associated with dose-dependent increases in zinc in cows [148, 149]. Additionally, superoxide dismutase (SOD) activity increases with zinc supplementation during IVM with bovine COCs, and although cumulus expansion is unaffected, the proportion of CGCs undergoing apoptosis is reduced [150]. Further, zinc supplementation increases blastocyst rates for COCs, denuded oocytes, and denuded oocytes cultured with CGCs, indicating that the mechanism that employs extracellular zinc to reduce reactive oxygen species (ROS) is not entirely dependent on the somatic cells [150]. Supplemental zinc during culture of COC from small antral follicles, containing transcriptionally active bovine oocytes, enhances both transcription and competence to undergo maturation, suggesting that zinc during oocyte growth is also important for full developmental potential [151]. Both zinc supplementation and hormones such as estradiol, LH, and FSH modulate the zinc quota and expression of zinc transporters in both CGCs and oocytes during bovine IVM [152]. This dynamic regulation is similar to the changes observed in mouse oocytes [56, 104] and highlights the tight regulation of zinc ions during maturation.

Zinc supplementation during IVM and IVF in mice displays similar trends with a dose-dependent increase in MII formation,

successful fertilization, and blastocyst development [153]. Conversely, zinc chelation increases ROS that is associated with reduced MII oocyte quality and impaired embryonic development [154]. While zinc supplementation during IVM of porcine oocytes does not enhance the percentage of MII oocytes, blastocyst formation rate following IVF is increased in association with reduced ROS and increased GSH [155]. Similarly, increasing concentrations of zinc supplementation of yak oocytes enhances the percentage of embryos reaching the blastocyst stage and is also associated with reduced ROS and increased intracellular GSH concentrations and SOD activity in matured oocytes [156]. By contrast, zinc supplementation during IVM in horses does not improve oocyte maturation, cleavage, or blastocyst formation [157]. Together, these studies indicate a dose-dependent relationship between zinc supplementation during IVM and reduced ROS, which is conserved across many mammalian species. However, excess zinc supplementation during IVM may curtail the positive benefits of zinc as the percentage of embryos that reach the blastocyst stage appear to slightly reduce after ~1–1.5 mg/mL zinc [148, 155, 156]. As these experiments did not use zinc concentrations above 2 mg/mL during IVM, additional studies are necessary to assess the concentrations of zinc that become toxic.

While zinc supplementation during IVM improves IVF outcomes, how extracellular zinc enhances the buffering of ROS in oocytes and COCs is unknown. Zinc has both protective and toxic properties dependent on the concentration and cell type. In primary neuronal astrocytes derived from rats, high zinc concentrations in the media inhibited GSH redox cycling to promote ROS and cell death [158]. Conversely, human adult retinal pigment epithelial cells or rat vascular endothelial cells treated with similar zinc concentrations were protected against oxidative stress by elevating GSH through Nrf2-mediated transcription of glutamate-cysteine ligase, the rate-limiting enzyme for GSH synthesis [159, 160]. For oocytes, the latter mechanism is likely taking place as depletion or disruption of Nrf2 reduces CCNB1/CDK1 expression and disrupts spindle organization, chromosomal alignment, and polar body extrusion similar to zinc depletion [161, 162].

Zinc in preimplantation development

Upon completion of meiosis II and successful fertilization, the mammalian egg transitions into a preimplantation embryo that shifts from meiotic to mitotic division. Initially, cell division occurs without the intervening growth of daughter cells, resulting in smaller and smaller embryonic cells that fit within the ZP. Maternally derived transcripts stored in the egg prior to oocyte maturation provide the necessary gene products for early embryogenesis until embryonic genome activation (two- to eight-cell stage). As the embryo develops and increases in cell count, compaction and cell polarization define the outside and inside of the embryo, while localized signaling determines the cell differentiation and lineage.

Total bound and labile zinc is an order of magnitude higher (~4- to 10-fold) in the preimplantation embryo compared to iron and copper, with the total amount of zinc atoms at similar levels to the GV oocyte [163]. This high concentration of zinc persists throughout early embryo development from the one-cell to at least the eight-cell stage. Zinc is primarily localized to discrete punctate structures that are enriched around the cortex [104, 163]. It is therefore no surprise that zinc status is just as important for embryogenesis as it is for oocyte maturation and fertilization.

In vitro culture of embryos with TPEN at the one-, two-, and four-cell stages results in the dose-dependent developmental arrest and

a reduction in blastomere quality that was not reversible following culture in TPEN-free media [163]. The phenotypes observed were associated with more tightly packed DNA, particularly around the nucleolus, and a reduction in global transcriptional activity [163]. It is possible that ZD or zinc chelation, particularly during the one-cell stage, may inhibit the activation of the embryonic genome that occurs around the two-cell stage in mice. Then, as maternally derived transcripts are continuously degraded, the overall translational capacity would be reduced and lead to developmental arrest. Alternatively, maternally derived transcripts may still be available, but an insufficient zinc pool instead reduces the translational capacity through the inhibition of ribosomal RNA synthesis by RNA polymerase I [3]. Culture of blastocysts in a low-zinc environment results in a somewhat delayed formation of inner cell mass (ICM) and visceral endoderm, which may be due to the increased oxidative stress and apoptosis [164].

In vivo, preimplantation mouse embryos are unable to recover from maternal ZD even if zinc is later supplemented, with a critical period of zinc requirement appearing to be between fertilization and the four-cell stage [165, 166]. Additionally, early blastocyst development and competence is adversely affected following the preconception ZD in mice [6]. Even an acute bout (3–5 days) of preconception ZD delayed blastocyst progression through reduced expression of imprinting genes, such as the growth-promoting genes, *Igf2* and *H19* [6]. Further, preconception ZD compromises the later stages of embryo development, placentation, and survival throughout pregnancy likely through altered epigenetic programming of the oocyte and/or early embryo (see discussion below) [6, 88].

Epigenetic programming of oocytes and early embryos

Germ cells and early embryos are particularly susceptible to epigenetic disruption. Oocytes acquire high levels of chromatin methylation as ovulation approaches [167–169], but after fertilization, the paternal genome is actively demethylated, while the maternal genome is passively demethylated [170–173]. As preimplantation embryo development proceeds, there is extensive re-methylation of both the ICM and trophectoderm (TE) lineages [170, 173, 174]. However, TE lineages have a dramatically different epigenetic landscape from the epiblast cells with extensive methylation at CpG islands and promoter regions, particularly the promoters of pluripotency genes such as *Oct4* and *Nanog* [175–177]. In both lineages, DNA methylation is necessary to establish the imprinted (monoallelic) gene expression [178] and for silencing of damaging repetitive elements [179, 180]. The DNA methylation is maintained at imprinted control regions (ICRs) [181–183] and on imprinted alleles through the de novo methylation in the zygote/early embryo [184].

Failure to maintain imprinted DNA methylation at imprint control regions [170, 185] causes epigenetic defects that are transmitted to the offspring [6, 186, 187]. Importantly, imprinted genes are crucial for placenta development, and their dysregulation is associated with impaired function and adverse pregnancy outcomes [188, 189]. Previous work using acute dietary ZD shows that preconception ZD causes chromatin hypomethylation in oocytes [6]. Interestingly, supplementation of zinc-depleted oocytes with SAM during in vitro maturation restores DNA methylation and partially rescues fertilization defects [6], suggesting that methyl donors could help correct the epigenetic defects caused by lack of zinc. A similar phenomenon occurs in mice supplemented with a methyl donor-rich diet that increases DNA methylation and alters gene expression at specific loci [190].

Epigenetic effects of ZD on postimplantation embryo and placenta development

Zinc deficiency prior to fertilization can have lasting effects on the development of the embryo and supporting tissues, even when the zinc intake becomes adequate during pregnancy (Figure 3). Acute ZD during the preovulatory period results in reduced DNA and histone methylation with an increased abundance of repetitive elements in the mouse GV oocytes [6]. These epigenetic defects are associated with growth delays and defects in embryonic and placental development later in pregnancy. For example, following acute ZD, embryos are 31% smaller in size with a high 46% mortality rate on embryonic day 10.5 (E10.5) [88]. These defects were specific to ZD of the embryo itself rather than the uterus as embryo transfer of ZD embryos to females fed with a normal, zinc-sufficient diet had reduced the implantation rates and smaller embryos following implantation [88]. Strikingly, as few as 5 days on a ZD diet prior to ovulation reduces the embryonic implantation rate by up to 75%, with successfully implanting embryos reduced in size by 38% and trophoblast outgrowth area reduced by 40% [88]. Preconception ZD also has long-lasting effects on the developing embryo as the fetal contribution to the placenta is significantly reduced and defects in limb buds and neural tube development were noticed even when zinc was sufficient throughout the pregnancy [88]. The impairments in neural tube development caused by preconception ZD could be related to the increase in cell death and reduction in ectoderm formation observed in zinc-deficient embryos [164] and to a reduction in working memory seen during gestational ZD [191].

Effects of ZD during gestation on fetal development

Zinc is an essential micronutrient known to be of paramount importance for successful fetal development during pregnancy. Severe skeletal and cardiac abnormalities occur when there is not enough zinc in the diet [36, 88, 192–197]. Zinc deficieny during gestation also results in defects in neural tube development as fetal neural progenitor cell proliferation is impaired by decreased phosphorylation of ERK1/2 [198]. However, proliferation of a neuroblast-like cell line challenged with ZD can be recovered with the inhibition of protein phosphatase 2 [198]. While similar abnormalities are observed in embryos that underwent preovulatory ZD or ZD during gestation, whether the same signaling mechanisms are involved is not certain. What is certain however is that ZD disrupts cellular proliferation and increases apoptosis, particularly in cell types that have high proliferation rates, such as along the neural tube. This observation is sensible as zinc serves as a cofactor in many metalloenzymes involved in DNA transcription and protein synthesis [3, 199–201].

Interestingly, the plasma membrane transporter, Znt1, which belongs to the Slc30 family of transporters involved in zinc efflux from the cytoplasm, is essential for embryonic development as mouse homozygous *Znt1* knockout (*Znt1*$^{-/-}$) in embryos result in death by embryonic day 9 (E9) [202]. While normal embryonic growth occurs during early embryogenesis, morphological changes are observed in the *Znt1*$^{-/-}$ animals following implantation around embryonic day 7 (E7). These changes are attributed to the failure of zinc passage through the embryo-derived visceral yolk sac and extraembryonic tissues to supply zinc to the embryo [202]. Additionally, zinc
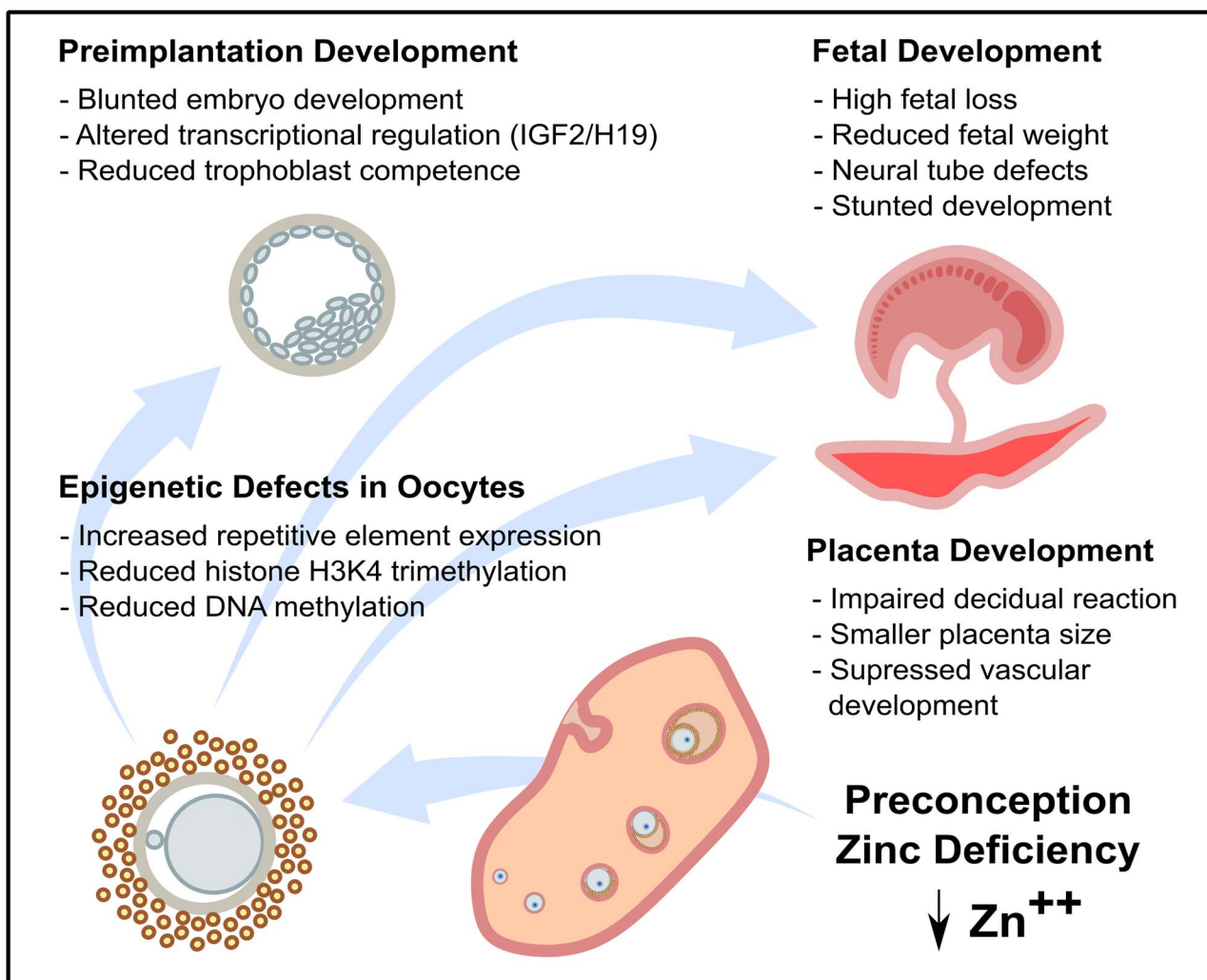
Figure 3. Epigenetic defects caused by preconception ZD. A 3–5 day period of dietary ZD is sufficient to cause a reduction in DNA and histone methylation and increased abundance of transcripts from the repetitive elements in the oocyte. These defects are associated with impaired preimplantation development, including reduced abundance of *Igf2/H19* transcripts and impaired trophoblast differentiation. However, even later in pregnancy after normal dietary zinc status is restored, defects in placenta formation, fetal survival, and fetal development persist, suggesting a long-lasting impairment of developmental potential.

supplementation to the mothers of the *Znt1*$^{-/-}$ mice does not rescue the E7–E9 morphological changes and death, suggesting *Znt1* is the critical mediator of zinc flux to the postimplantation embryo [202]. Furthermore, a higher proportion of *Znt1*$^{+/-}$ heterozygotes display delayed development, and higher rates of offspring with abnormalities occur when the mothers are challenged with ZD during pregnancy [202].

During postimplantation development, the embryo responds to ZD by upregulation of *Zip4* in the embryonic visceral yolk sac [203]. Homozygous knockout of *Zip4* is embryonically lethal in mice around mid-gestation at E9, while heterozygous embryos were more susceptible to ZD [204]. While the expression of other zinc transporters may also be regulated in response to ZD, *Zip1* and *Zip5* appear to have similar mRNA content regardless of the maternal zinc content [203].

Zinc deficiency in later but pre-placental embryonic stages has teratogenic effects, such as necrosis and gross malformation, particularly along the neural tube [205–207]. These teratogenic effects are also observed following exposure to chemicals that affect zinc

homeostasis [208, 209]. After placenta formation, zinc transporter expression within the tissue responds to dietary zinc uptake; however, these changes do not appear to save the embryo from severe insults of ZD [210].

Zinc and placenta development

The importance of zinc for placenta development and function is relatively unexplored but is potentially significant. There is evidence that both preconception and pregnancy zinc status are important for proper placenta function. Insufficient zinc in the diet could be a major contributing factor in poor placenta development and subsequent adverse pregnancy outcomes. For example, there is a strong association between ZD and risk of pre-eclampsia [39, 211–214], and ZD in pregnant rats during the last third of gestation produces fetuses with smaller brains and livers, while the placentas were less affected [215]. Recent findings demonstrate that marginal ZD causes a compensatory change to the labyrinth zone of the placenta and in maternal cardiovascular adaptations during pregnancy
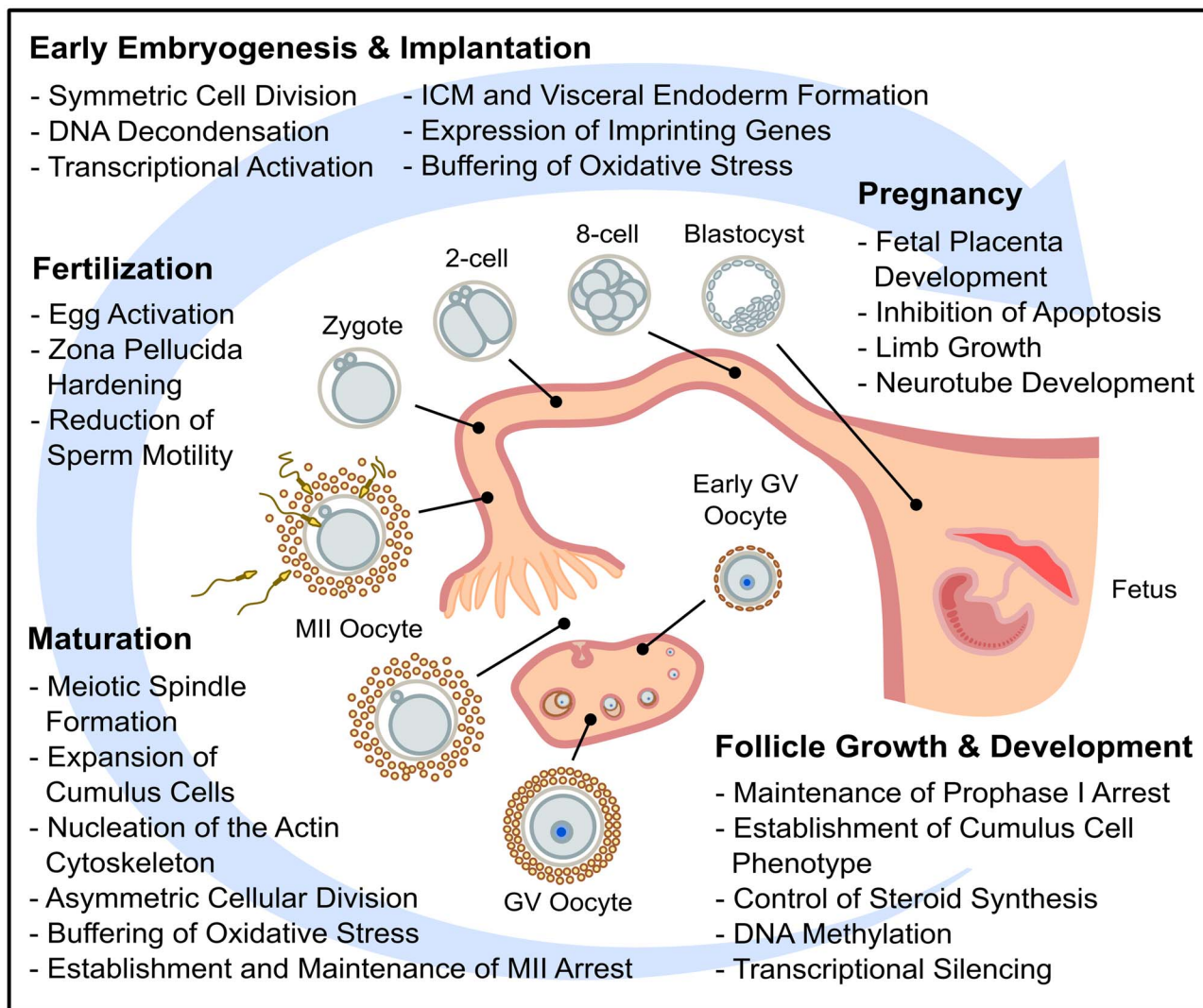
Figure 4. Summary of the events and processes in mammalian female reproduction that zinc is known to play a role.

in mice [216]. Recent work in mice shows that preconception ZD for 3–5 days impairs the trophoblast proliferation, implantation, and placenta development [6, 88]. The reduction in placental mass appears to be specific to a reduction in the area occupied by the fetal labyrinth zone, which is also associated with a reduction of the expression of many key placental genes [88].

Maternal to fetal zinc transport is essential for fetal growth and appears to be regulated by the umbilical blood flow [217], the presence of zinc-binding ligands that include albumin [218], metallothionein [219], an unidentified low molecular weight placental zinc-binding protein [220], and a zinc/potassium exchanger [221]. Zinc content is greater in the maternal blood and mid-disk region compared to the fetus [222–224] and so requires active processes of delivering zinc to the fetus. There are 24 known zinc transport proteins, and of these, mRNA for *Znt1, Znt2, Znt4,* and *Znt5* but not *Znt3* have been detected in human placentas, with *ZnT1* and *ZnT5* protein expressed along the apical membrane of the placental syncytiotrophoblast [210, 225]. The *Znt1-7* transcripts have also been quantified in mouse placentas, however, *Znt3* is lowly expressed and is only detectable at higher PCR cycles [226]. Importantly, the expression of both *Znt1* and *Znt5* respond to the dietary zinc

availability in mouse placentas and may therefore significantly contribute to the maintenance of fetal zinc content [210, 225]. The *Zip1* is also differentially expressed in the mouse placenta in response to dietary zinc [210]. While Zip4 is associated with inherited ZD disorders and is detected in the mouse embryonic visceral yolk sac [203], it is not expressed in the human placenta [225]. Given the many roles of the placenta (endocrine, metabolic, and developmental) and the widespread involvement of zinc in these processes, it is likely that we have only begun to understand the importance of zinc in placental development and function.

Perspectives, conclusions, and translational relevance

Although zinc was first recognized as a critical nutrient in mammalian female reproduction in the 1940s [24], recent discoveries over the last decade have greatly expanded our knowledge of the role of zinc in a multitude of reproductive processes, including oocyte growth and maturation, fertilization, epigenetic programing, and subsequent embryonic, fetal and placental development (Figure 4) [6, 10, 76, 87, 88, 104, 125, 127]. The wide-ranging effects of zinc are

mediated by the many zinc-binding proteins present in mammalian cells. By one estimate, up to 10% of human proteins are known or predicted to bind zinc ions [227]. It will be a challenge, in the years to come, to sort out the many pathways regulated by zinc ions in the ovary. Nevertheless, it is already clear that the active and dynamic movements of zinc ions, particularly in and out of the oocyte and zygote, are important signals regulating gamete function and embryo development. One of the current most clinically relevant zinc discoveries is the use of measuring zinc efflux during IVF to evaluate the developmental potential of individual oocytes. Assessing activated eggs by the magnitude of their zinc spark events could be a useful tool in selecting embryos with a high likelihood of implantation success. Additionally, supplementation of zinc in the IVM and IVF media for human oocytes has the potential to improve the outcomes of both techniques.

Given the important functions of zinc in female reproduction, it is not surprising that a lack of zinc in the diet (3–5 days preconception) or during in vitro maturation severely impairs fertility in mice though combined disruption of meiosis, fertilization, preimplantation, and postimplantation development. Likewise, in humans, dietary ZD severely impairs reproduction. The similarities and differences in zinc signaling in rodents versus humans or other mammals remains to be fully revealed, but the extensive commonalities shown in Table 1 strongly suggest that zinc ions are important for multiple conserved reproductive processes. Whether these are an important consideration in human reproduction remains to be determined. Zinc deficiency is common in many parts of the world [22], especially in pregnant women and in disadvantaged populations in the USA and abroad [228–230]. An estimated 82% of pregnant women worldwide do not consume the recommended dietary allowance for zinc [231]. Thus, the mechanisms of zinc action revealed in animals and the prevalence of ZD in humans underscore a pressing need to understand the significance of zinc and the consequences of ZD to human reproductive health.

Conflict of interest

The authors have declared that no conflict of interest exists.

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