A Story of Birth and Death: mRNA Translation and Clearance at the onset of Maternal-to-Zygotic Transition in Mammals

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Abstract

In mammals, maternal-to-zygotic transition (MZT), or oocyte-to-embryo transition, begins with oocyte meiotic resumption due to the sequential translational activation and destabilization of dormant maternal transcripts stored in the ooplasm. It then continues with the elimination of maternal transcripts during oocyte maturation and fertilization and ends with the full transcriptional activation of the zygotic genome during embryonic development. A hallmark of MZT in mammals is its reliance on translation and the utilization of stored RNAs and proteins, rather than *de novo* transcription of genes, to sustain meiotic maturation and early development. Impaired maternal mRNA clearance at the onset of MZT prevents zygotic genome activation and caused early arrest of developing embryos. In this review, we discuss recent advances in our knowledge of the mechanisms whereby mRNA translation and degradation are controlled by cytoplasmic polyadenylation and deadenylation which set up the competence of maturing oocyte to accomplish MZT. The emphasis of this review is on the mouse as a model organism for mammals and BTG4 as a licensing factor of MZT under the translational control of the MAPK cascade.

Introduction

The journey from a fertilized oocyte to a living organism begins with one of the most intriguing cellular program transformations in biology: remodeling of the fertilized oocyte into a totipotent zygote. In mammalian oocytes, maternal mRNAs are transcribed and stored during oocyte growth. They support oocyte meiotic maturation and early embryo development, but undergo general decay after meiotic resumption. In contrast, the zygotic genome is transcriptionally activated only in the later zygotic to 2-cell stage. In all animals, the transition from the maternal to zygotic mode of developmental control is called maternal-to-zygotic transition (MZT) [1].

In mammalian species, this transition initiates even before fertilization, at the final stage of oocyte development. Follicles are functional units of the mammalian ovary and each follicle consists of an oocyte surrounded by one or more layers of somatic cells [2]. A neonatal ovary is endowed with several million primordial follicles. Of those dormant primordial follicles that survive and are recruited into the growing follicle population, very few are able to form preovulatory follicles [3, 4]. In mammalian ovarian follicles, immature oocytes are arrested at the diplotene stage of meiosis I, which is also called the germinal vesicle (GV) stage, due to the existence of a vascularized nucleus. Along this lengthy developmental continuum, oocyte volume progressively increases and the oocytes synthesize and store large quantities of dormant mRNAs, which will later drive their re-entry into meiosis [5]. However, fully grown oocytes in preovulatory follicles are transcriptionally silent during the period from prior to meiotic resumption until after fertilization [6]. Most transcriptional reactivation takes place at the 2- to 8-cell stage in mammalian species, including humans [7, 8]. A luteinizing hormone surge at estrus triggers oocyte meiotic resumption, which is characterized by germinal vesicle breakdown (GVBD) in preovulatory follicles, followed by chromosome alignment and spindle organization at prometaphase I [9-11]. Subsequently, oocytes extrude a first polar body (PB1), spontaneously enter meiosis II, and are arrested again at metaphase II (MII), while awaiting fertilization. In maturing mammalian oocytes, massive maternal mRNA degradation is initiated as early as the meiotic resumption stage [12]. Meiotic cell cycle–coupled mRNA destabilization is considered an

immediate prologue to MZT in mammals and is a prerequisite for zygotic genome activation (ZGA) in early embryos, but studies of its cellular function and physiological importance have been inconclusive.

In this review, we define MZT as the period that begins at oocyte meiotic resumption with the translational activation of dormant maternal transcripts that encode major regulators of MZT, continues through the elimination of maternal transcripts during oocyte maturation and fertilization, and ends with the full transcriptional activation of the zygotic genome during embryonic development. We will discuss recent advances in research on the mechanisms whereby mRNA translation and degradation are controlled by cytoplasmic polyadenylation and deadenylation at the onset of MZT, with an emphasis on the mouse as a model organism for mammals. As recognized by researchers of various model systems, it is very difficult to separate the studies of MZT from those of oocyte meiotic maturation. Particularly, regulation of maternal mRNA translation and degradation mainly occurs in maturing oocytes rather than in fertilized eggs, but these mechanisms are essential for the oocyte and zygote to build up competence to accomplish MZT. The starting point of the MZT is oocyte activation from meiotic arrest rather than fertilization. In a historical review article by Tadros and Lipshitz, the authors stated: "In *D. melanogaster*, the elimination of maternal transcripts begins immediately upon egg activation, prior to and independently of any zygotic input. This also appears to be true in the majority of model organisms in which transcript elimination has been studied" [1].

To highlight state-of-the-art findings in this field, only major studies published in the last 3 years are discussed. To compare MZT regulation among different animal groups, known mechanisms of regulating mRNA translation and clearance in other model organisms, such as fruit fly, zebrafish, and African clawed frog, are also introduced.

Translational repression and activation of maternal mRNAs in mammalian oocytes

In vertebrates, fully grown GV-stage-arrested oocytes contain a large number of translationally dormant maternal mRNAs, with a short poly(A) tail of 20 to 40 nucleotides. Upon meiotic maturation, the poly(A) tail of many maternal mRNAs is elongated to 80–250 residues and the mRNAs are translationally activated. During the first meiotic division, shortly after GVBD, translational hotspots develop in the chromosomal area and in a region that was previously surrounded the nucleus. These distinct translational hotspots are controlled by the activity of the mTOR–eIF4F pathway [13]. These temporally translated proteins play key roles in meiotic spindle assembly, MII arrest maintenance, and mRNA clearance during MZT.

The mechanisms responsible for translational repression of dormant maternal mRNAs in GV oocytes have been extensively studied in *Drosophila* and *Xenopus*. For example, the *Drosophila* RNA binding protein (RBP), Glorund, binds an element in 3′-untranslated regions (3′-UTRs) during oogenesis to maintain repression in the ooplasm [14]. In the early embryo, another RBP, Smaug, is synthesized and binds a different 3′-UTR cis-element to repress translation by recruiting Cup, a eukaryotic translation initiation factor-4E (eIF4E)-binding protein that prevents the recruitment of the 40S ribosomal subunit [15]. It has been demonstrated in *Xenopus* oocytes, that the timing of translational activation for each transcript depends on a combinatorial code of cis-elements in the 3′-UTR that are able to recruit different sets of RBPs [16, 17]. These motifs include the polyadenylation signal (PAS, also known as hexanucleotide AAUAAA), which is bound by cleavage and polyadenylation specificity factor (CPSF) and cytoplasmic polyadenylation elements (CPEs, UUUUAU or UUUUAAU), which recruit CPE binding proteins (CPEBs) [18]. CPEBs have dual functions of deadenylation and polyadenylation. In fully grown GV oocytes, 3′-UTR-bound CPEB1 interacts with the poly(A)-specific ribonuclease, PARN and the polyadenylation machinery, including CPSF and GLD-2 poly(A) polymerase. PARN has a stronger catalytic activity than GLD-2 and therefore, it antagonizes GLD-2 to maintain transcript deadenylation [19, 20]. Upon meiotic resumption, the phosphorylation of CPEB1 by cell cycle-related kinases, including aurora A, CDK1, and ERK1/2, increases its affinity for CPSF, while decreasing its interaction with PARN, thus switching its function to the stimulation of cytoplasmic polyadenylation and translational activation [21].

Nonetheless, the mechanisms for controlling mRNA translation in mammalian oocytes are evidently different from those in *Xenopus* oocytes. Studies using *Gld2*-knockout mice (which are healthy and fertile) have concluded that this key poly(A) polymerase in *Xenopus* is dispensable in mouse oocytes, indicating that evolutionally divergent mechanisms are employed [22]. In addition, previous studies have reported the composition and regulation of protein complexes that mediate the translational repression and activation of CPE-containing mRNAs by maternal factors, such as PARN [23] [24], Maskin [25], and Symplekin [26] in *Xenopus* oocytes. However, the involvement of their mammalian homologs in oocyte maturation and MZT has not been reported.

Recently Yang and colleagues demonstrate that the timing of *Ccnb1* mRNA translation in mouse oocytes depends on the presence of transcripts with different length of 3′-untranslated regions (UTRs). These different splicing variants show distinct temporal patterns of translational activation or repression and therefore provide an additional layer of translation control [27]. However, it remains unclear how these transcript variants are formed during oocyte growth and how many maternal transcripts are regulated in the similar manner. Using 3′-UTRs of three model mRNAs, mouse *Cpeb1*, *Btg4*, and *Cnot6l*, which are subject to different translation patterns during oogenesis and present a different arrangement of CPEs and PAS [28-30], Dai and colleagues deciphered the combinatorial code that controls developmental stage-specific translation during meiotic maturation in mammalian oocytes [31]. Their findings were as follows:

(i) Translation of a maternal transcript at the GV stage requires one or more PASs that are located distant from CPEs.

(ii) PASs distal and proximal to the 3′-end of transcripts, are equally effective in mediating translation at the GV stage if they are not close to CPEs. This observation reveals a previously unrecognized non-canonical mechanism by which proximal PASs mediate 3′-terminal polyadenylation remotely.

(iii) Both translational repression at the GV stage and activation after GVBD require at least one CPE adjacent to the PAS.

(iv) The number and position of CPEs, in relation to PASs within the 3′-UTR of a given transcript, determines its repression efficiency in GV oocytes.

(v) The potential involvement of CPSF components has been investigated in *Xenopus* oocytes, but never been proved in mammalian oocyte maturation and MZT [21]. By overexpressing a dominant negative form of CPSF4, which is a PAS-binding subunit of CPSF complex [32, 33], this study demonstrated that normal CPSF function was required for cytoplasmic polyadenylation of maternal mRNAs in oocytes as well as meiotic cell cycle progression.

It still needs to be tested that it this code explains the translational behavior of most or all mRNAs in oocytes. This is technically difficult at current stage because the information regarding the dynamics of polyadenylation and translation of ALL maternal transcripts is lacking. Nevertheless, these studies help lay the foundation for future genome-wide studies to decipher the combinatorial codes in the 3'UTRs of maternal mRNAs that regulate their translational repression and/or activation from the GV stage to MZT in mammals. Despite recent progress in understanding translational control of mRNAs during mammalian MZT, a number of important questions remain unanswered:

(i) Although the elongation of poly(A) tails in the cytoplasm is essential for oogenesis, the poly(A) polymerases responsible for cytoplasmic polyadenylation in mammalian oocytes have not been identified. Several poly(A) polymerases responsible for cytoplasmic polyadenylation have been identified in yeast (Cid1), *C. elegans* (Gld-2), and *Xenopus* (xGld-2). Poly(A) polymerase D4 (PAPD4) is a mouse homologue of *Xenopus* xGLD-2. Despite the ubiquitous expression of PAPD4 in mouse tissues, *Papd4*-knockout mice were normal and healthy. Moreover, *Papd4* disruption did not affect poly(A) tail elongation in mouse oocytes. Thus, other PAPD family members, or other families of poly(A) polymerases, may play a redundant role with PAPD4.

(ii) Although the combinatorial code of CPE and PAS controlling the developmental stage-specific translation of maternal mRNAs during meiotic maturation has been recently deciphered, other 3´-UTR-binding factors originally reported in *Drosophila*, including Smaug, Musashi, and Pumilio, may also be involved in cytoplasmic translational regulation in mammals. The physiological importance of these factors in mice has also been reported, but their roles in forming a more

sophisticated combinatorial code that regulates 3′-UTR repression and activation need to be further investigated.

(iii) Mechanistic studies in *Xenopus* oocytes indicate that CPEB1 is a dual-function RNA-BP that, when not phosphorylated, recruits the poly(A) RNase, PARN, to deadenylate and repress maternal mRNAs [34]. CPEB1 also mediates translational repression of maternal mRNAs in GV oocytes by recruiting Maskin, which prevents the binding of CPSF to PASs in transcripts [34]. However, the potential functions of PARN and Maskin-like proteins in mammalian oocytes have not been investigated.

MAPK cascade couples translational activation of dormant mRNAs to meiotic resumption

Temporal and spatial regulation of cytoplasmic polyadenylation and translation are essential posttranscriptional control mechanisms during development and oogenesis [35]. Substantial work over the years has identified both cis- and trans-acting factors that regulate these processes. In mouse oocytes, maternal mRNAs are translated or repressed depending on the state of oogenesis. Despite these advances, our knowledge of the molecular details of these processes is far from complete.

Meanwhile, a parallel line of study has indicated that the mitogen-activated protein kinase (MAPK) cascade plays pivotal and evolutionarily conserved (from *C. elegans* to mouse) roles in regulating oocyte meiotic cell cycle progression [36, 37]. In the last 2 decades, numerous papers have reported that inhibition or knockout of extracellular signal-regulated kinases 1 and 2 (ERK1/2), two of the most-studied MAPK family members, in mammalian oocytes severely impairs microtubule organization and meiotic spindle assembly [38, 39]. Deletion of ERK1/2 or their upstream kinase, MOS, causes precocious sister chromatid separation and oocyte parthenogenetic activation [38, 40, 41]. However, the identity of the substrates of phosphorylation by ERK1/2 in mammalian oocytes remains elusive. Efforts have been made to identify the ERK1/2 phosphorylation substrates on meiotic spindles and chromosomes. MISS (MAPK interacting and spindle stabilizing) and DOC1R (deleted in oral cancer 1 related) are reported as ERK1/2 substrates that regulate microtubule organization of mouse MII oocytes [42, 43]. However, the physiological function of ERK1/2 in oocyte maturation

has not been sufficiently elucidated, considering the profound influence of the MAPK cascade on this process.

Recent advances have shed new light on the essential function of ERK1/2 in oocytes and have connected these kinases with translational activation of maternal mRNAs [44]. ERK1/2 activity was found to be indispensable for the accumulation of the MZT licensing factor, BTG4 (described in detail later) [30]. Follow-up studies revealed that the MAPK cascade is not only required for protein synthesis of many important maternal mRNAs, but is also the long-searched-for signal that couples the translational activation of dormant oocyte mRNAs with meiotic cell cycle progression [45].

Mechanistically, ERK1/2 triggers the phosphorylation of CPEB1 on two conserved serine/threonine residues (Ser-181 and Ser-207 in mice) at an early stage of oocyte meiotic resumption [45]. Phosphorylated CPEB1 is recognized and polyubiquitinated by the ubiquitin E3 ligase, CRL1-βTrCP. As a result, a large proportion (70–90%) of CPEB1 proteins undergo degradation in meiosis I [46]. This degradation causes a change in the CPEB/CPE ratio and results in translational activation of a broad spectrum of CPE-containing maternal mRNAs. Therefore, by activating CPEB1, ERK1/2 couples the translation of a series of maternal mRNAs to meiotic maturation. The translation products of these maternal mRNAs are required for meiotic divisions and MZT [29]. Overexpression of phosphorylation-site-mutated CPEB1 has a dominant negative effect in mouse oocytes and impairs the translation of these maternal transcripts, including *Tpx2*, *Ccnb1*, and *Btg4* [45]. Phenotypically, spindle assembly, chromosome alignment, and meiotic divisions are disrupted in these oocytes. These novel findings show that CPEB1 is one of the most important phosphorylation targets of ERK1/2 and that it mediates the major functions of ERK1/2 in oocyte meiosis, i.e., the translational activation of dormant maternal transcripts.

CNOT6L is the essential catalytic subunit of CCR4-NOT that mediates maternal mRNA degradation in mouse oocytes

This activation of translation is transient, because polyadenylated maternal mRNAs are quickly targeted for degradation. Approximately 90% of maternal mRNAs are degraded during MZT in mouse embryos. In the major pathway of mRNA degradation, shortening of

the poly(A) tail, or deadenylation, is the first and rate-limiting step [47]. Poly(A) tail shortening reduces the binding of poly(A) binding proteins (PABPs) and slows down translation [48].

An important enzyme complex involved in poly(A) shortening is the deadenylase, CCR4-NOT, which is conserved from yeast to humans [49, 50]. In addition to at least 6 non-catalytic subunits (a large scaffold subunit, CNOT1 and 5 regulatory subunits with unknown function, CNOT2-4, CNOT9, and CNOT10), it contains a RING domain E3 ubiquitin ligase subunit (CNOT4), with unclear biological function [51], and two distinct subunits with ribonuclease activity: a subunit homologous to yeast CAF1, characterized by a DEDD (Asp-Glu-Asp-Asp) domain, and a yeast CCR4 homolog, containing an endonuclease-exonuclease-phosphatase (EEP) domain. In vertebrate cells, the complexity of the protein complex is further increased by the presence of two CAF1 paralogs (CNOT7 or CNOT8) and two CCR4 paralogs (CNOT6 or CNOT6L) [52]. It remains unclear whether the CCR4-NOT complex is a functional entity or whether it consists of subunits or small subcomplexes that are functionally active outside of the complex. Duplication of the genes encoding CCR4-NOT catalytic subunits in vertebrates suggests that the paralogs (*Cnot6* versus *Cnot6l*; *Cnot7* versus *Cnot8*) may have tissue-specific and/or target-specialized roles in mRNA deadenylation. Moreover, the coexistence of the deadenylases, CNOT6/6L and CNOT7/8, in vertebrates raises the question of whether their functions are complementary or redundant.

CNOT6L, instead of its close homolog, CNOT6, is preferentially expressed in fully grown mouse oocytes. Recently, our group generated a *Cnot6l*-knockout mouse strain and found that this gene is not essential for survival or homeostasis [29]. The only defect observed in *Cnot6l*-null mice is severe subfertility in females. Deletion of *Cnot6l* impairs the deadenylation and degradation of a subset of maternal mRNAs during oocyte maturation. Oocytes develop normally in the ovaries of *Cnot6l*-null mice and are capable of resuming meiotic maturation, characterized by GVBD. However, the majority (~90%) of *Cnot6l*-deleted oocytes fail to release PB1 and develop to the MII stage. These oocytes

contain distorted and multipolar spindles at both the MI and MII stage, such that the chromosomes were not aligned at the equatorial plates.

Fewer transcripts are degraded during the GV-MII transition in *Cnot6l*$^{-/-}$ oocytes, when compared with wild-type oocytes. Among the significantly degraded transcripts during the GV-MII transition in WT oocytes, many are stabilized in *Cnot6l*$^{-/-}$ oocytes at the MII stage, indicating that CNOT6L plays an important and indispensable role in mRNA degradation during GV-MII transition. Moreover, RNA sequencing of polysome-bound mRNAs revealed that significantly more transcripts show increased polysome-binding in *Cnot6l*$^{-/-}$ oocytes at the MI and MII stages. Importantly, approximately two-thirds of stabilized transcripts show increased polysome binding in *Cnot6l*$^{-/-}$ oocytes after GVBD, indicating that they are actively translated. Over-translation of these undegraded mRNAs causes microtubule–chromosome organization defects, which lead to activation of the spindle assembly checkpoint and meiotic cell cycle arrest at prometaphase I. Therefore, CNOT6L-dependent decay of specific maternal mRNAs is a prerequisite for the meiotic maturation of an oocyte. Recently, another group independently reported reduced fertility and impaired maternal transcript clearance in *Cnot6l*-knockout mice [53]. Vieus and Clarke describe that a subgroup of mRNAs are deadenylated in growing oocytes, become polyadenylated during early maturation and then deadenylated during late maturation. They also showed that the deadenylase, CNOT6, is present in cortical foci of oocytes and regulates deadenylation of these mRNAs [54]. These studies provide direct genetic evidence that the CCR4-NOT complex is crucial for maternal mRNA decay in mammalian oocytes during meiotic maturation.

Therefore, it seems that a specific biological function of maternal transcript clearance following meiotic resumption is to downregulate RNA binding proteins, the protein translation machinery, and their regulatory factors in oocytes, thereby keeping the ooplasm in a state conducive to meiotic cell cycle progression. In particular, this state involves acentriolar spindle assembly and homologous chromosome separation, which are unique to meiosis in oocytes.

RNA binding protein, ZFP36L2, is a CNOT6L adaptor that triggers maternal mRNA degradation

CNOT6L and other subunits of the CCR4-NOT complex do not contain an RNA-binding domain. Therefore, CNOT6L must use an RNA-binding protein as an adaptor to target maternal mRNAs that are scheduled for degradation during oocyte meiotic maturation. In *Xenopus* oocytes, mRNAs regulated by deadenylation are characterized by the (A+U)-rich element (ARE) sequence, AUUUA. An ARE-binding protein, C3H-4, recruits the CCR4-NOT deadenylase complex to ARE-containing mRNAs and causes shortening of poly(A) tails. C3H-4 accumulates in MI oocytes and ablation of this protein causes meiotic arrest. Mechanistically, an 'early' wave of cytoplasmic polyadenylation at the onset of oocyte meiotic resumption activates the synthesis of C3H-4, which in turn recruits the CCR4–NOT complex to maternal transcripts containing both CPEs and AREs in their 3′-UTRs. This negative feedback loop is required for metaphase exit into interkinesis and for meiotic progression of *Xenopus* oocytes.

Zinc finger protein 36-like 2 (ZFP36L2) is the mouse homolog of *Xenopus* C3H-4. A natural mutation of mouse *Zfp36l2*, in which the N-terminal 29 amino acid residues are deleted, leads to defects in oocyte maturation and ovulation [55]. A recent study using an oocyte-specific *Zfp36l2*-knockout mouse model indicates that oocyte-specific loss of ZFP36L2 prevents ARE-dependent mRNA decay and global transcriptional silencing in fully grown oocytes [56]. It also causes the formation of MII oocytes with defective spindles, abnormally large polar bodies, and fertilization defects [56]. ZFP36L2 also mediates chromatin modification and global transcriptional silencing in oocytes. ZFP36L2 downregulates mRNAs encoding transcription and chromatin modification regulators, including a large group of mRNAs for histone demethylases, targeting H3K4 and H3K9. Oocytes lacking ZFP36L2 fail to accumulate histone methylation at H3K4 and H3K9, which is associated with genome transcription silencing [56]. These results indicate that ZFP36L2-dependent mRNA degradation acts as a developmental switch and triggers wide-spread shifts in chromatin modification and global transcription during oocyte growth.

However, it is not known whether ZFP36L2 also plays a direct role in meiotic-resumption-coupled maternal mRNA decay at a later stage.

Coimmunoprecipitation experiments have revealed that ZFP36L2 preferentially binds to CNOT6L, rather than CNOT7 and recruits CNOT6L to target ARE-containing maternal mRNAs during murine oocyte maturation. Moreover, small-interfering-RNA–mediated *Zfp36l2* depletion in fully grown mouse oocytes does not affect meiotic resumption, but it does impair PB1 emission and spindle assembly [29]. These phenotypes are similar to those observed in *Cnot6l*-null oocytes. Consistent with these observation, AREs are enriched in the 3′-UTR of transcripts that are degraded during the normal GV-MII transition, as well as the maternal transcripts that are stabilized after *Cnot6l* knockout [29]. Collectively, these results suggest that ZFP36L2 associates with CNOT6L and functions as a CCR4–NOT adaptor in triggering the degradation of ARE-containing transcripts at the meiosis I stage of oocyte maturation,.

BTG4 functions as a meiotic cell cycle-coupled MZT licensing factor

CCR4-NOT deadenylase does not directly interact with RNAs, but relies on adaptor RNA-binding proteins to target poly(A) tails of cytoplasmic transcripts. In addition to ZFP36L2, the BTG/TOB family of proteins comprises another group of CCR4-NOT adaptors that function by recruiting CCR4-NOT to target mRNAs [57, 58]. The BTG/TOB family is unique to metazoans. Six family members, namely *Tob1, Tob2,* and *Btg1-4*, are found in vertebrates, including *Xenopus*, zebrafish and mouse. The conserved N-terminal BTG domain contains 2 regions, Box-A and Box-B, which display notable sequence conservation (Fig. 1). The function of the more divergent C-terminal regions is unclear. While ZFP36L2 is a CNOT6/6L-bound adaptor protein, all BTG and TOB proteins primarily interact with the CNOT7 or CNOT8 catalytic subunit of CCR4-NOT [59]. Previous studies have shown that overexpression of BTG and TOB family proteins in cultured cell lines inhibits cell proliferation and these proteins may function as tumor suppressors *in vivo* [60].

Among the BTG/TOB family members, only BTG4 is expressed exclusively in human and mouse oocytes and early embryos. Yu et al. (2016) [30] and Liu et al. (2016) [61] reported that BTG4 is the MZT licensing factor that had been long searched for in oocytes. *Btg4*$^{-/-}$ mice are viable and have normal body size. However, mouse embryos lacking BTG4 arrest at the 1–2 cell stage and *Btg4*-knockout female mice are infertile, whereas males exhibit normal fertility. *Btg4*$^{-/-}$ females displayed normal ovarian histology and ovulated MII oocytes, but zygotes derived from *Btg4*$^{-/-}$ females arrest at the 1- or 2-cell stage. A major biochemical consequence of the absence of BTG4 is a global delay in maternal mRNA degradation during MZT [62].

BTG4 interacts with the CNOT7 and CNOT8 subunits of the CCR4-NOT complex, similarly to other BTG family members. The interaction between BTG4 and the CCR4-NOT complex depends on multiple conserved amino acid residues within the BTG domain of mouse BTG4, particularly tryptophan-95 (Fig. 1). A mutation of tryptophan-95 to alanine (BTG4^{W95A}) abolishes the BTG4-CNOT7 and BTG4-CNOT8 interactions. The conserved lysine-203 residue in mouse CNOT7 is also essential for the interaction between BTG4 and CNOT7. Furthermore, a genetic knock-in mouse strain carrying a homologous *Btg4*^{W95A} mutant has similar phenotypes to *Btg4*$^{-/-}$ mice, with fertilized oocytes arrested at the 1- or 2-cell stage. In addition, maternal transcripts that are degraded during normal oocyte maturation are stabilized in oocytes and zygotes expressing only BTG4^{W95A} [30]. These results provide *in vivo* evidence that the biological function of BTG4 in triggering maternal mRNA decay during MZT is mediated by the CCR4-NOT deadenylase complex. In addition to maternal transcript clearance, the first wave of embryonic gene transcription at the 2-cell stage is also blocked in embryos derived from *Btg4*-knockout females. Therefore, the phenotype of this mouse model provides direct evidence that global maternal mRNA clearance during maternal-zygotic transition is a prerequisite for ZGA in mammals.

Multiple lines of evidence have suggested that BTG proteins may function as adaptors in recruiting mRNAs to CCR4-NOT deadenylase for their deadenylation. BTG4 does not contain an RNA binding domain itself, but is capable of binding to the mRNA 5′-cap binding protein, eIF4E, and PABPs, including both the nuclear PABP (PABPN1) and the

oocyte-specific cytoplasmic PABP (PAPBC1L), as detected by co-immunoprecipitation (eIF4E and PABPC1L) and yeast two hybrid (PABPN1) experiments [61, 63]. The identification of interactions between BTG4 and the translation initiation factor, eIF4E, suggests that temporal control of maternal mRNA degradation may be coupled with translation. Actively translating mRNAs form closed loops in the cytoplasm when the 5′-cap-bound translation initiation complex (containing eIF4E) interacts with PABPs [30]. By interacting with both 5′- and 3′-RNA binding proteins, BTG4 recruits CCR4-NOT precisely to the loop junction and triggers mRNA decay during MZT.

Knockout mice have been reported for all BTG/TOB family members, but none of these mice show appreciable phenotypes, execept that *Btg4* is exclusively expressed in oocytes and is indispensable for female fertility [30, 58]. This suggests that BTG- and TOB-encoding genes play highly redundant roles in somatic tissues and in male germ cells. However, FOG-3, the only BTG/TOB family member in *C. elegans*, is a key regulator of male germ cell development [64]. The only defect observed in *fog-3* mutants is that the hermaphorodite germline only develops into oocytes and not sperm cells. It appears that the regulation of reproduction by BTG- and TOB-encoding genes has been conserved in evolution, although different species use different developmental strategies and BTG4 has been specialized to regulate MZT in mammals.

Mechanisms that couple mRNA decay with meiotic cell cycle progression

Maternal transcripts remain relatively stable in GV-stage arrested oocytes. However, the degradation of a large number of transcripts begins immediately upon meiotic resumption and finishes strictly at the 2-cell stage. The key mechanism that couples maternal mRNA destablization with meiotic cell cycle progression is the meiotic maturation-coupled translation of maternal *Btg4* and *Cnot7* mRNAs.

Although *Btg4* and *Cnot7* mRNA levels are high in GV oocytes, expression of their proteins is only detected after GVBD, reaching a maximal level at the MII stage and then

rapidly decreasing after the 2-cell stage. *Cnot6l* transcripts also undergo translational activation after GVBD. The 3′-UTR of mammalian *Btg4* and *Cnot6l* mRNAs contains multiple putative CPEs, which are crucial for translation [30, 31]. After meiotic resumption, the intrinsic oocyte MAPK cascade and CPEB1 trigger the polyadenylation and translation of *Btg4*, *Cnot7*, and *Cnot6l* transcripts stored in fully grown oocytes by targeting these CPEs [29, 45, 65]. The accumulated BTG4, CNOT7, and CNOT6L proteins, in turn, mediate maternal mRNA deadenylation and degradation. U0126, a specific inhibitor of the ERK1/2 upstream kinases, MEK1 and MEK2, blocks the translational activation of these transcripts during oocyte meiotic maturation. In oocyte-specific *Erk1/2*-knockout mice, BTG4 and CNOT7 proteins do not accumulate in oocytes at the MII stage [45]. Consequently, maternal mRNA decay is blocked. Therefore, an ERK1/2-triggered negative feedback regulation mechanism ensures transient, but not prolonged, translation of proteins during MZT (Fig. 2).

Although both CNOT6L and BTG4 accumulate in maturing oocytes in response to ERK1/2 activation and trigger maternal mRNA decay, knockout models of these two genes yield different phenotypes in oocytes. The majority of *Cnot6l*-null oocytes are arrested at prometaphase I due to impaired spindle assembly and PB1 emission, but maternal *Btg4*-knockout oocytes complete meiotic maturation and are arrested at 1- to 2-cell stages during embryonic development [29, 30]. At the molecular level, a greater number of maternal transcripts are stabilized in *Cnot6l*-null oocytes than in *Btg4*-null oocytes, particularly during the GV-to-MII transition. This phenomenon is consistent with the observation that *Cnot6l* deletion causes an earlier phenotype (incomplete meiotic maturation) than *Btg4* deletion (inability to accomplish MZT) (Fig. 2). Meiotic maturation defects have been reported in mouse oocytes after RNA-interference–based depletion of *Btg4*. However, this phenotype does not exist in *Btg4*-null oocytes matured *in vivo* or *in vitro* [63]. This discrepancy may occur because the phenotypes of knockout mice reflect physiological functions of an individual gene more accurately and more specifically than those obtained by RNA interference approaches.

The question remains why knockouts of *Cnot6l* and *Btg4* cause different phenotypes in mouse oocytes. A likely explanation is that CCR4-NOT deadenylase has more than one type

of adaptor in maturing oocytes. At early (prometaphase) and late (meiosis II) stages of oocyte maturation, ZFP36L2 and BTG4 associate with CNOT6L and CNOT7/8, respectively and function as alternative CCR4-NOT adaptors to triggering the developmental stage-specific degradation of maternal transcripts. The stepwise recruitment of different adaptors by different catalytic subunits tightly couples the degradation of maternal mRNAs to meiotic cell cycle progression and ensures that this process is irreversible.

Methylation (m6A) regulates maternal mRNA stability

N6-methyladenosine (m6A) is the most abundant internal mRNA modification [66]. m6A sites contain an RRACU (R=G/A) consensus motif. Transcriptome analyses in human cells and mouse tissues has shown that m6A modifications are enriched near the start and stop codons and within long exons [67]. In mammals, the m6A modification is catalyzed by a multicomponent methyltransferase complex that includes methyltransferase-like 3 (METTL3), METTL14, and Wilms' tumor 1-associated protein (WTAP) [68]. It can be demethylated into adenosine by 2 known demethylases: fat mass and obesity-associated factor (FTO) and AlkB homologue 5 (ALKBH5) [69]. YTH-domain family member 2 and 3 (YTHDF2/3) have been identified in pull-down experiments using RNA probes that selectively bind m6A-methylated mRNAs [70]. The discovery of cellular m6A methyltransferases (writers), demethylases (erasers), and m6A binding proteins (readers) highlights the importance of m6A in basic biological functions and disease.

Most relevant to the focus of this review, methylation (m6A) of mRNAs has been found to regulate maternal mRNA stability in vertebrate oocytes. Over one-third of maternal zebrafish mRNAs can be m6A modified [71]. Germ cell-specific inactivation of the m6A methyltransferases, *Mettl3* or *Mettl14*, within proliferating male germ cells using *Ddx4-Cre*, causes loss of m6A and depletion of spermatogenic stem cells. m6A depletion dysregulates the translation of transcripts that are required for SSC proliferation and differentiation . Deletion of *Mettl3* and *Mettl14* in advanced germ cells using *Stra8-GFPCre* disrupts spermiogenesis [72]. Spermatids from double-mutant mice exhibit impaired translation of haploid-specific genes that are essential for spermiogenesis. This study highlights crucial

roles of mRNA m6A modification in male germline development, potentially ensuring coordinated translation at different stages of spermatogenesis. However, while *Mettl3* and *Mettl14* are also deleted in oocytes of these *Cre*-transgenic mouse lines, female fertility does not seem to be affected. Therefore, the identity and function of m6A methyltransferases during oogenesis and MZT remain to be specifically investigated.

Although the role of m6A methyltransferases in female germ cells remains obscure, studies in zebrafish and mice have shown that the clearance of maternal mRNAs is facilitated by an m6A-binding protein, YTHDF2, which is a cytoplasmic protein expressed at all stages of mammalian gametogenesis. Removal of YTHDF2 in zebrafish embryos decelerates the decay of m6A-modified maternal mRNAs, hence causing downregulation of zygotic transcripts and impeding zygotic genome activation [71]. Similarly, in mice, YTHDF2 deficiency is partially permissive and results in female-specific infertility. The loss of YTHDF2 in mouse oocytes results in the failure to establish a normal maternal transcriptome during oocyte development, with enrichment for the YTHDF2-binding consensus and m6A in upregulated genes [73]. YTHDF2 appears to promote the decay of maternal, but not zygotic, mRNAs during early development and therefore, regulates transcript dosage across oocyte maturation, which is essential for generating mature oocytes that are competent to sustain early zygotic development.

However, neither the studies in zebrafish nor the studies in mice explain the biochemichal mechanisms whereby the m6A modification affects maternal transcript stability. The interplay between m6A reader proteins and the RNA degradation machinary has not been investigated. More importantly, given the fact that mRNA translation and degradation during mammalian MZT is tightly coupled with meiotic cell cycle progression, m6A generation and removal on maternal transcripts should also be linked to specific developmental stages. The exact role and stage-dependent regulation of m6A modification of maternal transcripts remains to be determined.

3′-uridylation as a novel mechanism to mediate maternal mRNA decay

In human cell lines, approximately one-fifth of transcripts with very short poly(A) tails (fewer than 25 nucleotides) are 3′-uridylated and this nucleotide addition optimizes the decay of the mRNA [74]. The shortening of the poly(A) tail to fewer than approximately 27 nucleotides results in the dissociation of the stabilizing PABPC and subsequent recruitment of terminal uridylyl transferases 4 and 7 (TUT4 and TUT7, respectively), which mediate mRNA 3′-uridylation. Conditional knockout of *Tut4* and *Tut7* in mice, using the oocyte-specifically expressed *Zp3-Cre* (*Tut4*$^{fl/fl}$;*Tut7*$^{fl/fl}$;*Zp3-Cre*), causes infertility [74]. Although the knockout mice ovulate normal numbers of oocytes, the majority of *Tut4*- and *Tut7*-null oocytes fail to complete meiosis I and several phenotypic abnormalities are observed, including abnormal spindle morphology with microtubule asters. The functions of TUT4 and TUT7, specifically their uridylation activity, are intrinsically required during oocyte growth to complete meiosis I and generate developmentally competent MII oocytes.

Transcriptome analyses have shown that 3′-terminal uridylation of mRNA, mediated by TUT4 and TUT7, sculpts the mouse maternal transcriptome by eliminating a subset of transcripts during oocyte development in growing follicles [74]. Differing from those in somatic cells, oocyte transcripts tend to contain a short poly(A) tail and a high proportion of terminal oligo-uridylation. Oocyte-specific knockout of *Tut4* and *Tut7* causes the accumulation of a cohort of maternal mRNAs with a high frequency of very short poly(A) tails and a loss of 3′-oligo-uridylation. By contrast, deficiency of TUT4 and TUT7 does not alter gene expression in a variety of somatic cells. Oligo-uridylation seems to be the primary signal that instructs transcript degradation in oocytes, as evidenced by the observation that TUT4 and TUT7 deletion abolishes oligo-uridylation in transcripts with short poly(A) tails. A poly(A) tail length of less than approximately 27 nucleotides leads to a loss of PABP occupancy and provides a recognition signal for TUT4 and TUT7 binding and subsequent 3′-uridylation [75].

In addition to their function in the development of GV-stage oocytes, terminal uridylyl transferases also execute programmed clearance of the maternal transcriptome in vertebrate

embryos during MZT [76]. As an evolutionarily conserved phenomenon, uridylation is induced at the onset of maternal mRNA clearance. When the homologs of mammalian TUT4 and TUT7 are depleted in zebrafish and *Xenopus*, maternal mRNA clearance is significantly delayed, leading to developmental defects during MZT, which occurs at the gastrulation stage in these species. Short-tailed mRNAs are selectively uridylated by TUT4 and TUT7 and highly uridylated transcripts are more rapidly degraded during MZT than those with unmodified poly(A) tails.

Recent studies have also identified a reader of RNA oligouridylation, DIS3L2, as a new player in a major pathway of RNA decay [77]. An oligo (U) tail is preferentially recognized by the the 3′-to-5′ exoribonuclease, DIS3L2, which mediates decapping and decay of the uridylated mRNA [78]. In somatic cells, many mRNAs normally undergo poly(A) tail removal by deadenylases as a first step prior to their decay by an exonucleolytic mechanism. However, the 3′–5′ decay of mRNAs can also occur in an exosome-independent and oligouridylation-dependent manner [79]. Deadenylated transcripts oligouridylated by TUT4/7 become ideal targets for DIS3L2. Despite these findings in cultured cells, the role of DIS3L2 in TUT4/7-mediated maternal mRNA decay during MZT has not been investigated in any model system.

Zygotic genome activation-coupled maternal mRNA decay

So far we discribed the role of maternal factors (such as MAPK cascade, CNOT6L, and BTG4) in regulating mRNA decay after oocyte meiotic resumption. On the other hand, there are two distinct RNA degradation pathways conserved in *Drosophila*, zebrafish, and *Xenopus*, either of which is insufficient for transcript elimination [47]. Only the concerted action of both pathways leads to elimination of transcripts with the correct timing, at the mid-blastular transition (MBT) [80]. The first pathway is maternally encoded, is targeted to specific classes of mRNAs through cis-acting elements in the 3′-UTR, and is also elucidated in mouse as described above. The second pathway is activated 2 h after fertilization and functions together with the maternal pathway to ensure that transcripts are effectively degraded by the MBT.

In *Drosophila* and zebrafish, maternal RNAs can be devided into four classes based on the pattern of degradation. The first class lacks both maternal and zygotic degradation elements and is stable. The second class is degraded exclusively by the maternal machinery prior to ZGA. A third class remains relatively stable before ZGA and is degraded exclusively by the zygotic machinery. The fourth class is continously degraded by the combined action of the maternal and zygotic machinery [80]. A later study suggests that 60% of *Drosophila* maternal transcripts is either cleared or significantly decreased, to below 50% of their initial level during the MZT: 14% exclusively by maternal activity, 22% only by zygotic activity, and 25% by the combined action of both mechanisms [81]. RNA sequencing data suggest that these subpopulations of maternal transcripts also exist in mouse and human oocytes and early embryos: a subset of maternal transcripts is degraded soon after meiotic resumption while others show later decreases that coincide with ZGA at the 2-cell stage (mouse) and 8-cell stage (human), respectively [29, 30, 82-84]. Therefore, evolutionarily highly conserved pathways may control transcript degradation throughout metazoa, opening up the possibility of exploiting the particular advantages of different model systems for future mechanistic and functional analysis.

Studies in *Drosophila*, zebrafish, and frog all indicate that the zygotically expressed micro-RNAs (miRNAs) are key components of the ZGA-dependent maternal mRNA decay pathway. Specific miRNAs promote the deadenylation and decay of hundreds of target mRNAs and thus sharpen and accelerate the MZT. For example, zebrafish miR-430 is expressed at the onset of zygotic transcription and facilitates the deadenylation and clearance of maternal mRNAs during early embryogenesis [85]. Its ortholog, miR-427 and miR-18, which share the 5′-proximal seed sequence AAGUGC with miR-430, are highly expressed in early embryos of *Xenopus laevis* and trigger the deadenylation and subsequent degradation of maternal transcripts [86, 87]. In contrast, the *Drosophila* miR-309 cluster, none of whose members carry the AAGUGC seed sequence, has been identified as a zygotic degradation activity that destabilizes several hundred maternal mRNAs [88]. Importantly, the maternal and the zygotic degradation activities are intimately linked in *Drosophila*: Smaug, which is a key component of the maternal degradation activity, controls the onset of ZGA, including the

transcription of the miR-309 cluster. Zygotically encoded degradation activity then feedback to eliminate maternal *smaug* mRNAs at the end of MZT [47, 89].

In mouse oocytes and embryos, however, there is not yet any evidence for an mRNA clearance role for zygotically synthesized miRNAs. On the contrary, there are data suggest that the maternal miRNAs are not functionally active in mouse oocyte and early embryos: oocyte-specific knockout of *Dgcr8*, which encodes an RNA-binding protein specifically required for miRNA processing, does not cause any abnormalities of oocyte maturation, MZT, and preimplantation development; the mRNA profiles of wild-type and *Dgcr8* null oocytes were essentially identical [90]. Therefore, it remains unclear if ZGA is a prerequisite for maternal mRNA clearance in early embryos of mammalian species, or if zygotically expressed mRNAs and proteins, rather than miRNAs, contribute to the ZGA-associated maternal mRNA decay activity in mammals.

Perspective

Despite the recent progress described in this review, there are many open questions remaining to be answered regarding the regulation of mammalian MZT. A limited number of maternal-effect genes that regulate oocyte mRNA translation and degradation have been identified in mice. For example:

1) Zygote arrest 1 (*Zar1*) has been identified in mice and is considered "the first identified oocyte-specific maternal-effect gene that functions during MZT". Female mice null for *Zar1* are infertile, because embryogenesis is blocked at the 1-cell stage [91, 92]. The ZAR1 protein contains a conserved 3CxxC zinc finger domain and is considered to be an RNA-binding protein. Studies in zebrafish and *Xenopus* oocytes suggest that ZAR1 functions as a repressor of maternal mRNA translation [93-95], but the underlying mechanism is unclear.

2) It is interesting to note that in *Xenopus* oocytes, PARN, a cap-dependent deadenylase, is sequestered in the nucleus and following nuclear membrane breakdown, is released

into the cytoplasm, where it is involved in maternal mRNA degradation [96]. This alternative strategy of sequestering deadenylases would provide an additional safeguard to ensure maternal mRNA longevity. The role of PARN, if any, in the degradation of maternal mRNAs in mouse oocytes is not known.

3) In *Drosophila* and *Xenopus* oocytes, multiple 3′-UTR binding factors, including Smaug [89, 97], Musashi [98], Pumilio [99], and Brain tumor [100, 101], have been reported to regulate developmental stage-dependent and transcript-specific cytoplasmic translational repression or activation. Knockout of their homologous genes in mice causes significant developmental and reproductive defects [102, 103], suggesting that these factors may also be of physiological importance in mammalian MZT. However, the interplay of these RNA-binding proteins with the core signalling and machinery of polyadenylation or deadenylation in mouse oocytes, such as the MAPK cascade, CPEB1, CPSF, and CCR4-NOT, has not been investigated.

With the sophistication of genome editing techiniques, it is now possible to edit the mouse genome much more efficiently [65, 104]. Therefore, it is convenient to test the *in vivo* function of poorly studied genes. Equally important, optimized RNA sequencing techniques provide powerful tools to investigate RNA dynamics and behavior at the whole transcriptome level [82, 83]. Because mouse oocytes and zygotes contain only a very small amount of RNA and protein, many traditional techniques are not applicable. Modified high-throughput analysis methods have greatly decreased the threshold of input sample quantities, making these techniques applicable to the study of mammalian MZT [84]. For example, single-cell RNA sequencing techniques allow researchers to monitor transcriptomal changes in single oocytes or embryo blastomeres [105]. To understand how the mRNA tail is regulated during MZT, tail sequencing (TAIL-seq) has been performed on *Drosophila*, zebrafish, and even mouse embryos [76, 106]. RNA immunoprecipitation sequencing (RIP-seq) enables the identification of groups of RNAs that bind to a given protein or transcripts containing specific modifications, such as m6A [71, 107]. The identification of new MZT regulators and wide application of these new methods will accelarate the accumulation of our knowledge of MZT in mammals.

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Figures

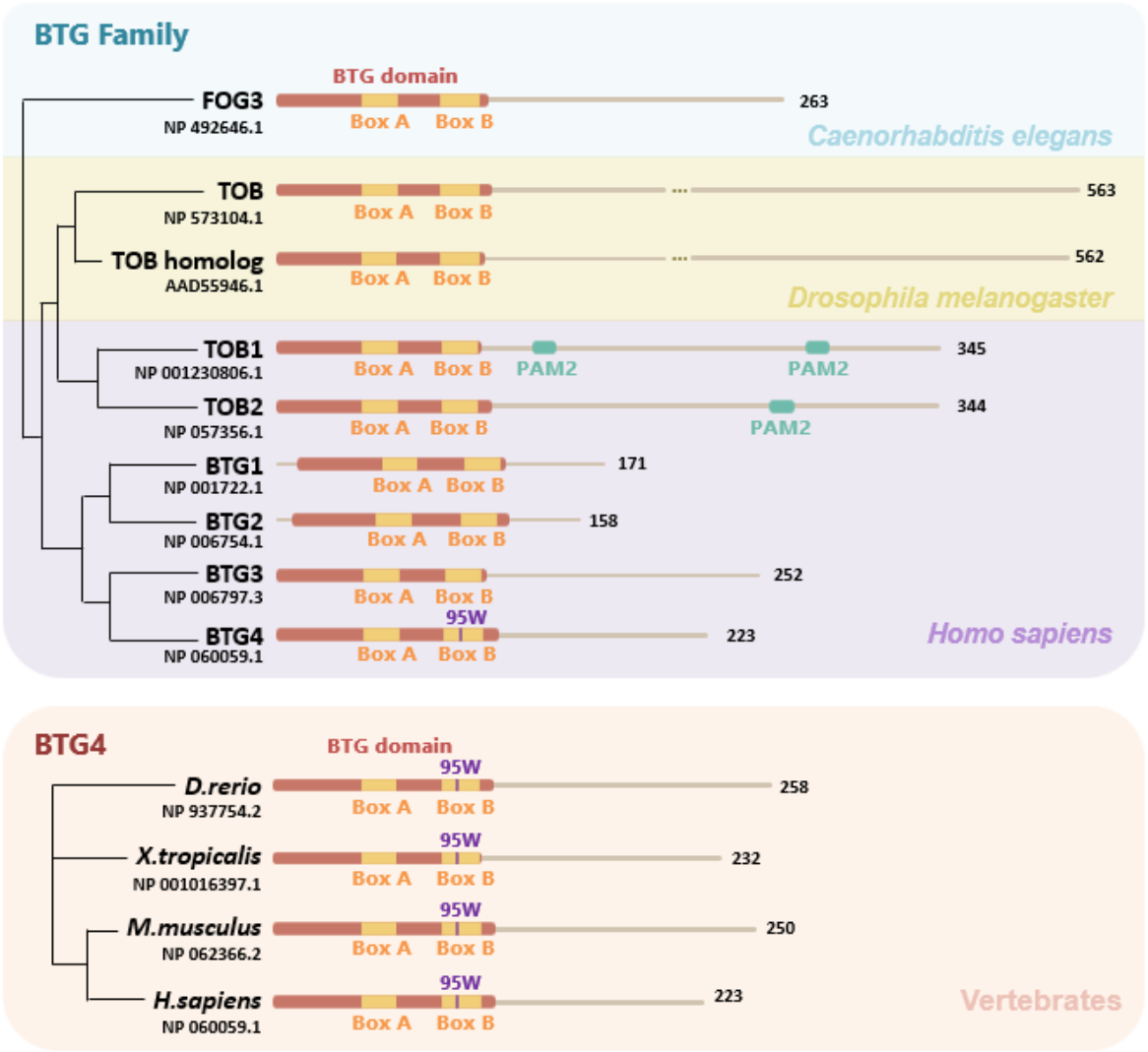

Figure 1: An overview of TOB/BTG family proteins. A: TOB/BTG family proteins of different animal species. The diagram, based on amino acid sequences, shows pair-wise relationships in the TOB/BTG family generated be Vector NTI. Schematic representations of the TOB/BTG family members highlights the conserved BTG domain (red) and the conserved PAM2 (green) motifs in TOB1/2 and BTG1/2, respectively, **B:** BTG4 proteins of different vertebrate species. The conserved W95 residue, which is essential for the *in vivo* function of BTG4, is indicated.

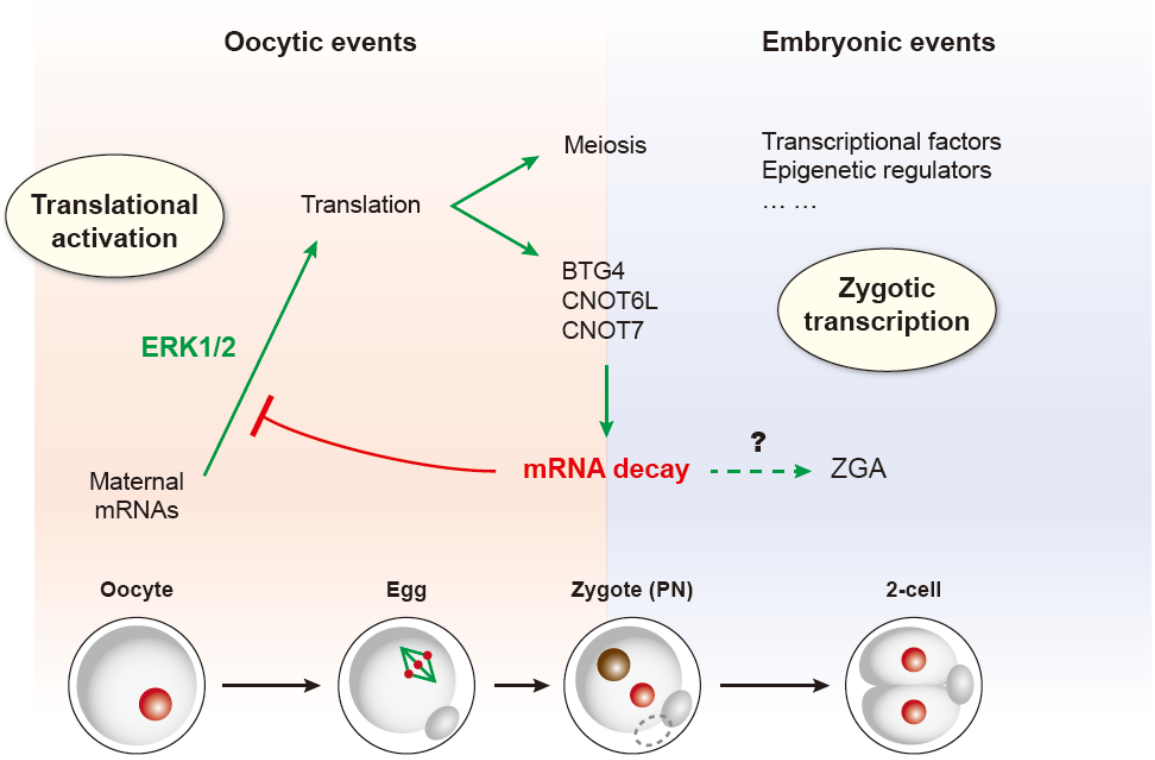

Figure 2: Feedback regulation of maternal mRNA translation and degradation during MZT in mice. Shortly after the onset of oocyte meiotic resumption, ERK1/2 is activated by the upstream kinases. Activated ERK1/2 trigger polyadenylation and translational activation of a wide spectrum of maternal mRNAs, including those encoding CNOT6L and CNOT7, the catalytic subunit of the CCR4-NOT complex, as well as the MZT licensing factor BTG4. These translational products of maternal mRNAs in turn target maternal transcripts for sequential degradation in oocytes and zygotes, therefore forming a negative feedback loop that prevents overtranslation of maternal transcripts during MZT. This biochemical process decreases global translation levels in oocytes and is essential for meiotic maturation. On the other hand, BTG4-mediated maternal mRNA decay is a prerequisite for zygotic genome activation for unclarified reasons (question mark). After sufficient clearance of maternal transcripts, zygotic genome resumes transcriptional activity, which involves the functions of multiple transcriptional factors and epigenetic regulators.