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Letter to the Editor

BTG4 is a key regulator for maternal mRNA clearance during mouse early embryogenesis

Dear Editor,

The maternal to zygotic transition (MZT) is a crucial process in the early development of almost all animals, during which maternal mRNAs are degraded and the zygotic genome is activated (Li et al., 2013). How maternal mRNAs are degraded is one of the long-standing questions in the field of developmental and reproductive biology. Recently, high-throughput sequencing and genetic studies have determined that the elimination of maternal mRNAs is accomplished by two modes: the first mode is dependent on maternally encoded transcripts, while the second mode relies on zygotic transcription (Yartseva and Giraldez, 2015). The first mode is well characterized in *Drosophila*, in which the maternally encoded RNA-binding proteins SMAUG and PUMILIO are important mediators of maternal mRNA clearance (Semotok et al., 2005; Gerber et al., 2006). The second mode is exemplified by zygotically transcribed miR-430 in zebrafish that directly targets and triggers the deadenylation and subsequent clearance of maternal mRNAs (Giraldez et al., 2006). Despite these encouraging findings in model animals of lower species, the mechanisms governing the selective maternal mRNA clearance are still largely unclear in mammals.

Deadenylation is the first and often rate-limiting step accounting for mRNA turnover (Garneau et al., 2007). The CCR4–NOT complex plays predominant roles in mRNA deadenylation, whereby it is involved in the SMAUG- and PUMILIO-mediated maternal mRNA clearance in *Drosophila* (Semotok et al., 2005; Gerber et al., 2006). Previous studies have shown that the anti-proliferative Tob/BTG protein family members are important players in CCR4–NOT-mediated mRNA deadenylation and subsequent degradation (Winkler, 2010). To investigate the potential roles of Tob/BTG proteins during mouse MZT, we first systematically analyzed the expression patterns of all Tob/BTG members (*Tob1*, *Tob2*, and *Btg1–4*) during this process by using RNA-seq data sets that are publicly available. Strikingly, among all the six members, *Btg4*, the main functional domain of which is highly conserved among vertebrates, showed an absolutely dominant and specific expression pattern during the MZT (Supplementary Figures S1 and S2A).

Next, we examined the spatio-temporal expression pattern of *Btg4* by quantitative real-time PCR (qRT-PCR) and western blot. *Btg4* was exclusively present in ovaries and testes (Supplementary Figure S2B). In oocytes and preimplantation embryos, *Btg4* mRNA was highly expressed in germinal vesicle stage (GV) oocytes and gradually perished during mouse preimplantation development, decreasing by ~90% by the 2-cell stage (Figure 1A). At the protein level, however, the translation of *Btg4* was largely initiated in metaphase II (MII) oocytes and was peaked at the 1-cell (1C) stage (Figure 1B, Supplementary Figure S2C), depending on the cytoplasmic polyadenylation elements and polyadenylation hexanucleotide AAUAAA sequence in the 3′ UTR (Supplementary Figure S3).

To investigate the physiological role of *Btg4*, we depleted *Btg4* using the CRISPR/Cas9 system that targets the first exon, and a targeted frame-shift mutant with a 116-bp deletion (*Btg4*$^{-/-}$) was successfully obtained (Supplementary Figure S4A and B). qRT-PCR and western blot showed that *Btg4* was efficiently disrupted at both RNA and protein levels (Supplementary Figure S4C and D). Disruption of *Btg4* had no effect on mouse viability and the fertility of male mice, but led to the infertility of female mice (Figure 1C). Histological analysis of paraffin sections from wild-type (WT) and *Btg4*$^{-/-}$ ovaries and superovulation experiments demonstrated that the oogenesis and fertilization of *Btg4*$^{-/-}$ oocytes were grossly normal (Supplementary Figure S5), but the development of embryos from *Btg4*$^{-/-}$ female mice was arrested at 1–2-cell stage (Figure 1D), indicating essential roles of *Btg4* during the MZT of mouse early embryogenesis.

We further performed transcriptome analysis on WT and *Btg4*$^{-/-}$ GV oocytes, MII oocytes, and 1C embryos (Supplementary Tables S1–S3). Although the transcriptome profile of *Btg4*$^{-/-}$ GV oocytes was comparable to that of WT, >46% (6401/13770) and 20% (2898/14058) mRNAs were upregulated in *Btg4*$^{-/-}$ MII oocytes and the resulting 1C embryos, respectively (Figure 1E). These findings were consistent with the temporal protein expression pattern and thus the functions of *Btg4* during mouse preimplantation development. Moreover, all the 12 genes, generated by overlapping the top 50 significantly upregulated genes in MII oocytes and 1C embryos, could be well validated by qRT-PCR, except for one gene with a very low expression level (Figure 1F, Supplementary Figure S6).

To test whether the aberrant upregulation of maternal transcripts is caused by the inefficient deadenylation of maternal mRNAs, poly(A) tail length (PAT) assay (Supplementary Figure S7A) was performed with several representative transcripts that were abundant in GV oocytes but significantly reduced in MII oocytes (Ma et al., 2015). The results showed that these transcripts in *Btg4*$^{-/-}$ MII oocytes and the resulting 1C embryos failed to be deadenylated and were thus resistant to degradation (Figure 1G and Supplementary Figure S7B). As the CCR4–NOT complex

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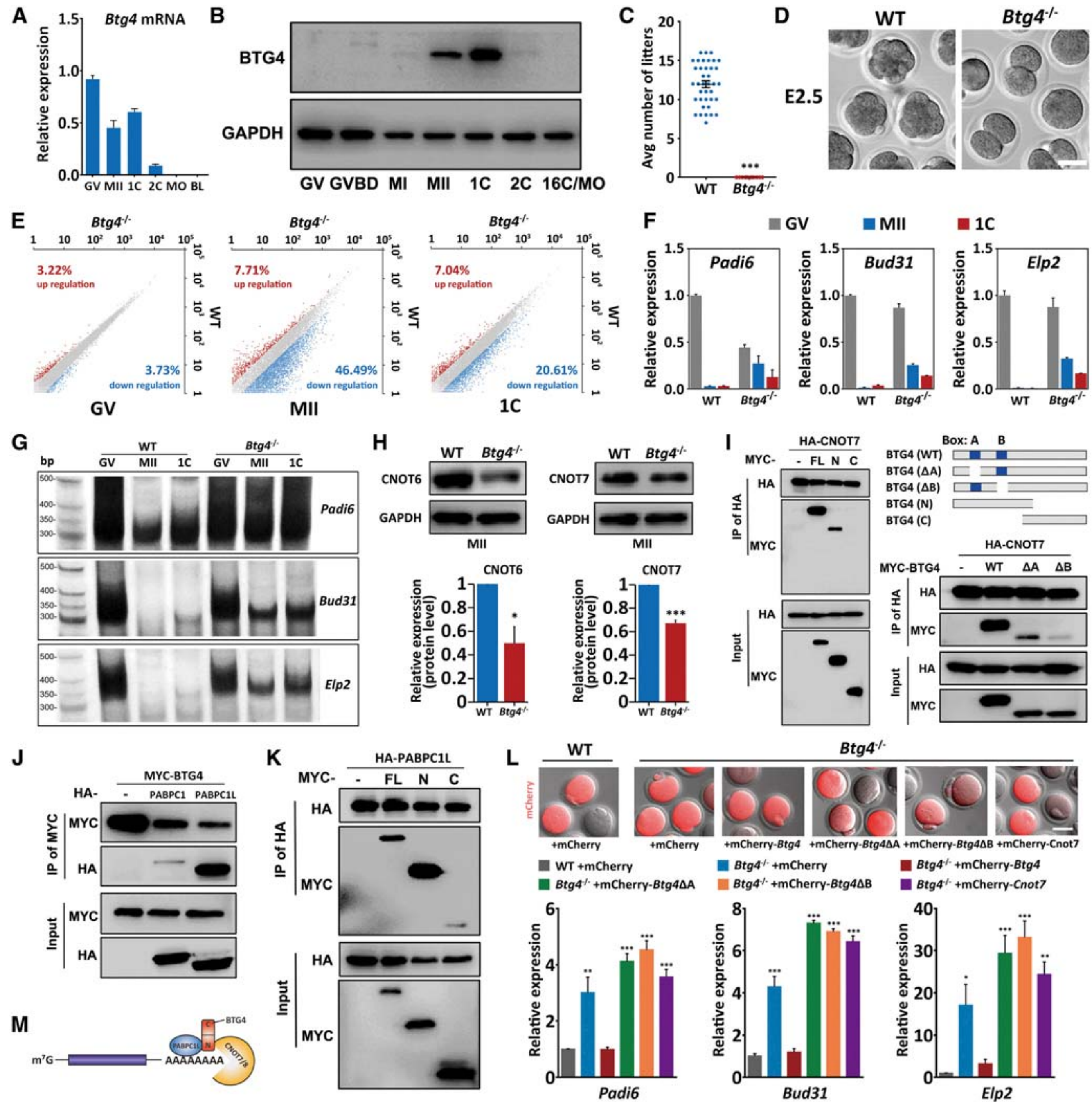

Figure 1 *Btg4* is involved in maternal mRNA deadenylation and subsequent decay. (**A**) qRT-PCR analysis of *Btg4* mRNA expression pattern in oocytes and preimplantation embryos. Error bars represent the SEM. (**B**) Western blot analysis of BTG4 protein expression pattern in oocytes and preimplantation embryos. GAPDH was used as a loading control. (**C**) Fertility assessment of WT and *Btg4*$^{-/-}$ female mice. WT (n = 38) and *Btg4*$^{-/-}$ (n = 12) female mice were mated with normal male mice over 3 months. The average pup numbers per litter were assessed. Error bars represent the SEM. (**D**) Developmental competence analysis of embryos derived from WT or *Btg4*$^{-/-}$ female mice. At E2.5, while embryos from WT female mice developed to the morula stage, the counterparts from *Btg4*$^{-/-}$ female mice were arrested at the 1–2-cell stage. Scale bar, 50 µm. (**E**) Whole transcriptome analysis demonstrates that the transcriptome profile was comparable in WT and *Btg4*$^{-/-}$ GV oocytes, but was significantly upregulated in *Btg4*$^{-/-}$ MII oocytes (46.49%) and 1C embryos (20.61%) (fold change ≥2, P < 0.05). (**F**) Representative upregulated transcripts (*Padi6*, *Bud31*, and *Elp2*) were analyzed in WT and *Btg4*$^{-/-}$ GV oocytes, MII oocytes, and 1C embryos by qRT-PCR. Error bars represent the SEM. (**G**) PAT assay of representative upregulated transcripts in *Btg4*$^{-/-}$ GV oocytes, MII oocytes, and 1C embryos compared with that in WT oocytes. (**H**) The effect of *Btg4* depletion on the expression of CNOT6 and CNOT7 in MII oocytes. The relative abundance was determined (the lower two panels) and the data were expressed as mean ± SEM (n = 3, *P < 0.05, ***P < 0.001). (**I**) Co-immunoprecipitation analysis of the interactions between CNOT7 and full-length (FL) BTG4, N-terminus, C-terminus, or truncated BTG4 with Box A or Box B depleted. (**J**) Co-immunoprecipitation analysis of the interactions between PABPC1, PABPC1L, and

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plays essential roles in mediating mRNA deadenylation, we tested the effects of *Btg4* knockout on this complex. Depletion of *Btg4* led to significant reduction of CNOT6 (~50%) and CNOT7 (~30%), two catalytic subunits of CCR4–NOT complex required for efficient deadenylation in the mRNA decay pathway, in MII oocytes (Figure 1H). The co-immunoprecipitation results showed that the N-terminal Box A and Box B, but not the C-terminus of BTG4, are responsible for the interaction with CNOT7 (Figure 1I), similar to other Tob/BTG family members in somatic cells (Winkler, 2010). Most importantly, the N-terminus of BTG4 interacts with PABPC1L (also known as ePAB) (Figure 1J and K), a well-known embryonic poly(A)-binding protein involved in mouse MZT (Ivshina et al., 2014). There was a very weak interaction between BTG4 and the ubiquitously expressed paralog PABPC1 (Figure 1J). Notably, the phenotype of aberrant upregulation of certain representative mRNAs in $Btg4^{-/-}$ oocytes could be rescued by full-length BTG4, but not BTG3 or truncated BTG4 with the N-terminal Box A or Box B depleted. Additionally, the overexpression of *Cnot7* alone could not restore the otherwise clearance of the representative maternal mRNAs (Figure 1L, Supplementary Figure S8).

While BTG3 binds to both PABPC1L and PABPC1 (Supplementary Figure S9), BTG4 specifically interacts with PABPC1L. In addition, only BTG4, but not BTG3 or CNOT7, can rescue the upregulation of maternal mRNAs in BTG4-null oocytes. Furthermore, the expression pattern of BTG4 is very similar to that of PABPC1L and CNOT7 (Supplementary Figures S10 and S11). All these data consistently suggest that BTG4 serves as a key adaptor of the CCR4–NOT complex via binding to an oocyte- and early-embryo-specific embryonic poly(A)-binding protein PABPC1L to target maternal mRNAs for their deadenylation and decay during mouse MZT. In the process of preparing our data for publishing, we noticed that similar observations were reported by Yu et al. (2016). While we found that depletion of BTG4 in MII oocytes led to significant downregulation of CNOT6 and CNOT7, the other group reported that the level of CNOT7 was hardly changed in the absence of BTG4 (Yu et al., 2016). This discrepancy might be ascribed to the difference between the antibodies used in these two studies. Altogether, our data demonstrate that BTG4 is a key regulator for the deadenylation and decay of thousands of maternal mRNAs during the MZT and bridges CCR4–NOT complex and PABPC1L to control maternal mRNA elimination in mammalian early embryonic development (Figure 1M). Considering the conservation, the BTG4–CCR4–NOT complex–PABPC1L involved in mRNA deadenylation might be a universal machinery for maternal mRNA decay in vertebrates, deficiency of which in humans might cause reproductive disorders. Our study gains new insights into the maternal mRNA elimination and significantly promotes the understanding of molecular mechanisms underlying the MZT in mammals.

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BTG4. The interaction between BTG4 and PABPC1L was much stronger than that between BTG4 and PABPC1. (**K**) Co-immunoprecipitation analysis of the interactions between PABPC1L and the N-terminus or C-terminus of BTG4. (**L**) FL *Btg4*, *Btg4* with Box A or Box B depleted, or *Cnot7* fused with mCherry mRNA (mCherry-*Btg4*/-*Btg4ΔA*/-*Btg4ΔB*/-*Cnot7*) or mCherry mRNA alone was, respectively, injected into WT and $Btg4^{-/-}$ GV oocytes. After 12 h, mCherry-positive oocytes were collected, and the representative transcripts (*Padi6*, *Bud31*, and *Elp2*) were examined. The aberrant upregulation of these transcripts was largely attenuated by FL *Btg4*, but not by *Btg4* lacking Box A or Box B, nor by *Cnot7* alone. The data were expressed as mean ± SEM (n = 3). (**M**) The working model of BTG4 involvement in maternal mRNA decay. BTG4 interacts with the poly(A)-binding protein PABPC1L in mouse oocytes and recruits the CCR4–NOT complex to deadenylate maternal mRNAs prior to their elimination.

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