The mRNA-destabilizing protein Tristetraprolin targets "meiosis arrester" *Nppc* mRNA in mammalian preovulatory follicles

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C-natriuretic peptide (CNP) and its receptor guanylyl cyclase, natriuretic peptide receptor 2 (NPR2), are key regulators of cyclic guanosine monophosphate (cGMP) homeostasis. The CNP-NPR2-cGMP signaling cascade plays an important role in the progression of oocyte meiosis, which is essential for fertility in female mammals. In preovulatory ovarian follicles, the luteinizing hormone (LH)-induced decrease in CNP and its encoding messenger RNA (mRNA) natriuretic peptide precursor C (*Nppc*) are a prerequisite for oocyte meiotic resumption. However, it has never been determined how LH decreases CNP/*Nppc*. In the present study, we identified that tristetraprolin (TTP), also known as zinc finger protein 36 (ZFP36), a ubiquitously expressed mRNA-destabilizing protein, is the critical mechanism that underlies the LH-induced decrease in *Nppc* mRNA. *Zfp36* mRNA was transiently up-regulated in mural granulosa cells (MGCs) in response to the LH surge. Loss- and gain-of-function analyses indicated that TTP is required for *Nppc* mRNA degradation in preovulatory MGCs by targeting the rare noncanonical AU-rich element harbored in the *Nppc* 3′ UTR. Moreover, MGC-specific knockout of *Zfp36*, as well as lentivirus-mediated knockdown in vivo, impaired the LH/hCG-induced *Nppc* mRNA decline and oocyte meiotic resumption. Furthermore, we found that LH/hCG activates *Zfp36*/TTP expression through the EGFR-ERK1/2–dependent pathway. Our findings reveal a functional role of TTP-induced mRNA degradation, a global posttranscriptional regulation mechanism, in orchestrating the progression of oocyte meiosis. We also provided a mechanism for understanding CNP-dependent cGMP homeostasis in diverse cellular processes.

TTP/Zfp36 | CNP/Nppc | mRNA degradation | oocyte meiosis | mural granulosa cells

Cyclic guanosine monophosphate (cGMP) is a ubiquitous second messenger that regulates myriads functions, such as the cell cycle, differentiation, apoptosis, and metabolism (1). During recent decades, C-natriuretic peptide (CNP), encoded by natriuretic peptide precursor C (*Nppc*), has emerged as a critical regulator of cGMP homeostasis by activating its transmembrane guanylyl cyclase receptor, natriuretic peptide receptor 2 (NPR2), which increases the intracellular cGMP concentration (2, 3). Increasing evidence from mouse models of genetic depletion or mutation of *Nppc* or *Npr2* and related clinical assays have indicated that the CNP-NPR2-cGMP signaling cascade is critical for the development and function of the cardiovascular (4, 5), skeletal (6, 7), nervous (8, 9), and female reproductive systems (10). In particular, CNP-NPR2-cGMP signaling is the central regulator of the progression of oocyte meiosis (11), the female germ cell division that is essential for haploid oocyte formation.

In mammals, oocytes are arrested at the diplotene stage of meiosis until the surge of luteinizing hormone (LH) restarts meiosis and initiates the ovulatory process. In preovulatory follicles, LH-induced CNP-NPR2-cGMP signaling inhibition is the most prominent

hallmark for meiotic resumption. Although LH also activates cGMP hydrolysis (12) and decreases guanylyl cyclase activity (13, 14) and NPR2 affinity for CNP in ovarian follicles (15), mouse models that prevent cGMP hydrolysis or/and NPR2 inactivation can reinitiate meiosis (12, 16), suggesting that these alterations are not indispensable for LH-induced meiotic resumption. These facts suggest a notable change upstream of the CNP-NPR2-cGMP signaling cascade; indeed, remarkable decreases in CNP and *Nppc* messenger RNA (mRNA) have been reported in mouse (14, 17–19), rat (13), pig (20), and human (17) follicles, and the time course of the *Nppc*/CNP decrease almost coincides with the resumption of meiosis (14). Previous studies have established the critical role of low-level CNP in decreasing cGMP and restarting meiotic progression. The incubation of isolated cumulus-oocyte complexes (COCs) with low-level CNP that recapitulates the follicular concentration after LH administration resumes meiosis; however, high levels of CNP maintain meiotic arrest (11, 20, 21). Thus, the LH-induced decrease in CNP levels is thought to be important for fine-tuning meiotic resumption (14, 22, 23). However, the mechanism underlying decreased CNP is unknown and thus represents an unanswered question in our understanding of the mechanism that controls

Significance

The fertility of female mammals depends upon the well-orchestrated progression of oocyte meiosis, the female germ cell division that is essential for haploid oocyte formation. In preovulatory follicles, how LH induces a decrease in CNP and its encoding mRNA *Nppc*, a prerequisite for oocyte meiotic resumption, remains an outstanding question to understand the mechanism that controls oocyte meiosis. The present study shows that TTP, an mRNA-destabilizing protein, is one of the regulatory components responsible for the LH-induced rapid decrease in *Nppc* mRNA and oocyte meiotic resumption. This finding not only highlights the importance of posttranscriptional regulation in the follicle periphery to fine-tune oocyte meiosis but also provides an insight into CNP-dependent cGMP homeostasis in other physiological systems.

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oocyte meiosis (22). Given that the turnover of CNP is very rapid (24), a decrease in *Nppc* mRNA could rapidly decrease the amount of CNP (14). In addition, the LH-induced decrease in CNP is independent of the natriuretic peptide clearance receptor NPR3 (18). Therefore, we focused on the possible mechanism underlying the LH-induced decrease in *Nppc* mRNA.

By reanalyzing a previously published transcriptome that presented rapid effects of LH on gene expression in the mural granulosa cells (MGCs) of mouse preovulatory follicles (25), we noticed that zinc finger protein 36 homolog (*Zfp36*) that encodes tristetraprolin (TTP) showed an expression pattern that was inversely related to the LH-induced decrease in *Nppc* mRNA. TTP is an RNA-binding protein that regulates mRNA stability by binding to AU-rich elements (AREs) in the 3′ untranslated regions (UTRs) of target mRNAs and destabilizes mRNA via poly(A) tail removal or deadenylation (26, 27). Furthermore, we noticed that *Nppc* mRNA is a representative ARE-containing transcript. Based on these findings, we hypothesized that TTP might target and degrade *Nppc* mRNA and thus could be the key factor underlying the LH-induced decrease in *Nppc* mRNA.

In the present study, we identified that TTP-induced *Nppc* mRNA degradation is a regulatory component of meiotic progression in preovulatory follicles. Notably, we reported that a rare noncanonical ARE motif mediated the TTP-induced destabilization of *Nppc* mRNA. Additionally, we found that LH transiently activated *Zfp36* via the epidermal growth factor receptor (EGFR)-extracellular regulated kinase (ERK)1/2-dependent pathway. Thus, focusing on the upstream part of the signaling cascade, the present study determined a mechanism by which CNP-NPR2-cGMP signaling is inactivated during meiotic resumption and ovulation processes and provides insight into CNP-dependent cGMP homeostasis in other physiological systems.

Results

Zfp36/TTP Is Transiently Up-Regulated in Response to the LH/hCG Surge and Targets the 3′ UTR of the *Nppc* mRNA.
To explore the mechanism underlying the LH-induced decrease in *Nppc* mRNA, we tested the previous notion that the LH-induced decrease in *Nppc* mRNA is a universal switch for CNP-NPR2-cGMP signaling inhibition. Female Institute of Cancer Research (ICR) mice were administered with human chorionic gonadotropin (hCG) to mimic the endogenous LH surge (14, 17–19), and then we detected the *Nppc* mRNA levels in MGCs and ovaries at various time points. We found that the *Nppc* mRNA levels decreased immediately in MGCs and ovaries to less than 50% by 2 h post-hCG and further declined to 10% by 5 h post-hCG (Fig. 1*A* and *SI Appendix*, Fig. S1*A*). Our findings, together with previous results obtained from mice with a different genetic background (17–19) and from pigs (20), indicated that the rapid decrease in the *Nppc* mRNA levels is a hallmark event common to different genotypes and species.

Next, to identify the potential factor controlling *Nppc* expression levels, we reanalyzed previously published transcriptome data that identified early response genes in MGCs at 1 h post-hCG. We noticed that the expression of the *Zfp36* gene, whose product, TTP, is responsible for degrading mRNAs that have AU-rich 3′ UTRs, was up-regulated by 3.3-fold within 1 h after LH/hCG administration (25). Given that the up-regulation of *Zfp36* coincided with the decrease in *Nppc* levels and that the *Nppc* 3′ UTR is considerably AU-rich, we hypothesized that *Zfp36*/TTP might be implicated in the decrease in *Nppc* mRNA. To this end, we isolated MGCs from preovulatory follicles at various times after injection with hCG and detected the expression dynamics of *Zfp36* using real-time reverse transcription quantitative PCR (RT-qPCR). In agreement with the results of previous transcriptome data (25), we found a transient and significant increase in *Zfp36* mRNA, by approximately sevenfold and tenfold at 1 and 2 h post-hCG, respectively, in MGCs and ovaries, followed by a rapid decline to the basal level (Fig. 1*B* and *SI Appendix*, Fig. S1*B*). In addition,

the transient activation of *Zfp36* mRNA in MGCs was accompanied by a transiently increased abundance of TTP at 2 to 4 h in response to hCG injection (Fig. 1*C*). Collectively, these results suggested that *Zfp36*/TTP is activated in response to hCG during a specific time window in preovulatory follicles. Moreover, by analyzing the previously published transcriptome data of ovarian MGCs pre- and post-LH/hCG administration from different species (28–31), we found that activation of *Zfp36*/TTP in preovulatory MGCs in response to the LH/hCG surge appears to be common in these species (*SI Appendix*, Fig. S2).

Next, immunohistochemical detection indicated that the LH/hCG-induced TTP is mainly localized in MGCs and cumulus cells (Fig. 1*D*), which overlaps with the distribution of *Nppc* mRNA in the preovulatory follicle (11). The spatiotemporally associated patterns, together with the negatively correlated expression dynamics between *Nppc* and *Zfp36*, suggested that *Zfp36*-encoded TTP might be an inducible repressor of *Nppc* mRNA in response to the LH/hCG surge. Therefore, we determined whether TTP could target the 3′ UTR of *Nppc* mRNA. Given that the high level of TTP protein appeared by 2 and 3 h post-hCG and *Nppc* mRNA was significantly decreased at 2 h post-hCG, we isolated preovulatory MGCs at 2 and 3 h post-hCG and used RNA immunoprecipitation (RIP) to test the binding of TTP to the *Nppc* mRNA. In a TTP-specific pulldown, *Nppc* mRNA, but not nontargeted beta actin (*Actb*) mRNA, was significantly enriched compared with the Immunoglobulin G (IgG) controls (Fig. 1 *E* and *F* and *SI Appendix*, Fig. S3*A*). The specificity of the interaction was confirmed by sequencing the RIP-qPCR amplicon.

Having confirmed the binding of TTP to the *Nppc* 3′ UTR, we next examined whether the *Nppc* 3′ UTR can mediate the TTP-induced mRNA destabilization. We generated luciferase constructs containing the full-length *Nppc* 3′ UTR, the tumor necrosis factor (*Tnf*) 3′ UTR, or the *Actb* 3′ UTR. The *Tnf* 3′ UTR was used as the targeted control because *Tnf* mRNA is a well-accepted target of TTP (32), whereas the *Actb* 3′ UTR was used as the nontargeted control (Fig. 1*G*). Humanembryonic kidney (HEK293) cells were cotransfected with the psiCHECK2 luciferase vector containing *Nppc* 3′ UTR or the control 3′ UTRs, together with a *Zfp36* overexpression vector or empty vector. Compared with cells transfected with the empty vector, overexpression of *Zfp36* significantly reduced the luciferase activity of the reporter containing the full-length *Nppc* 3′ UTR to the level comparable to that of the reporter containing full-length *Tnf* 3′ UTR but did not alter the luciferase activity of the reporter containing full-length *Actb* 3′ UTR (Fig. 1*G*). To further determine the role of *Nppc* 3′ UTR in mediating TTP-induced destabilization of target mRNA, we measured the luciferase mRNA stability after blocking transcription using actinomycin D (ActD), which is frequently used to detect mRNA stability by inhibiting de novo mRNA synthesis, thus facilitating the measurement of the remaining mRNA (33–35). The results showed that TTP overexpression significantly accelerated the destabilization of luciferase mRNA containing the full-length *Nppc* 3′ UTR to approximately a quarter of that of the control vector after 3 h (Fig. 1*H*), which is comparable to the rate of decline of *Nppc* mRNA in MGCs following the LH/hCG surge. The reliability of the mRNA stability assay was supported by the fact that TTP overexpression significantly degraded luciferase mRNA containing the *Tnf* 3′ UTR but not that containing the *Actb* 3′ UTR (*SI Appendix*, Fig. S3 *B* and *C*). These results indicated that TTP can destabilize the *Nppc* mRNA via its 3′ UTR. Similar results were also obtained using the bovine *NPPC* 3′ UTR (*SI Appendix*, Fig. S3 *D* and *E*). Collectively, these experiments demonstrated that *Zfp36*/TTP can be activated in preovulatory follicles in response to the LH/hCG surge and then targets the *Nppc* 3′ UTR, which might contribute to hCG-induced destabilization of *Nppc* mRNA.

TTP Targets the Noncanonical ARE Motif in the *Nppc* 3′ UTR.
Luciferase assays demonstrated that the full-length *Nppc* 3′ UTR contains functional repressive motifs that respond to TTP. AREs
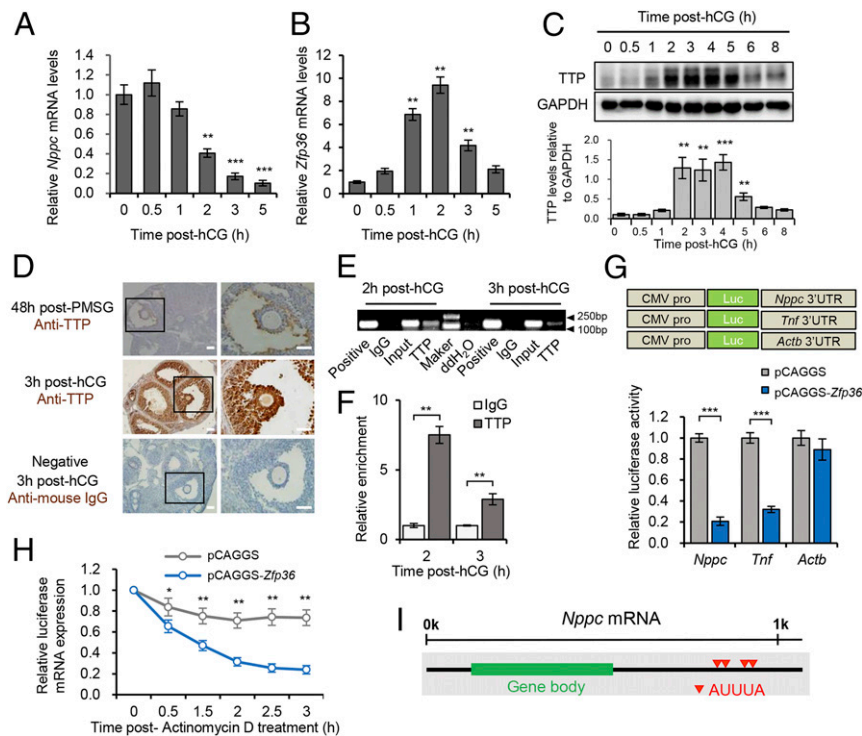
Fig. 1. TTP is up-regulated in response to the LH/hCG surge and targets the *Nppc* 3′ UTR. (*A* and *B*) RT-qPCR detection of the expression dynamics of *Nppc* (*A*) and *Zfp36* (*B*) mRNA in preovulatory MGCs in response to hCG administration. (*C*) Western blotting analysis for TTP in preovulatory MGCs at different time points after hCG administration. The upper and lower bands indicate phosphorylated (inactive) and unphosphorylated (active) forms of TTP, respectively. (*D*) Immunohistochemical staining of TTP in preovulatory follicles before and after hCG administration. (*Right*) Higher magnification of boxed regions. (Scale bar, 100 μm.) (*E*) RIP analysis for the direct binding of TTP to the *Nppc* 3′ UTR. The RIP sample was subjected to RT-PCR, and the amplified products were visualized using agarose gel electrophoresis. (*F*) Quantitative detection of the interaction between TTP and *Nppc* 3′ UTR by RIP–RT-qPCR. (*G*) Schematic diagram of the luciferase constructs containing full-length *Nppc* 3′ UTR, *Tnf* 3′ UTR (targeted control), or *Actb* 3′ UTR (nontargeted control). Relative luciferase activity of different luciferase vectors, which were cotransfected with a *Zfp36* overexpression vector or empty vector in HEK293 cells. (*H*) Measurement of the stability of the luciferase mRNA that contained the *Nppc* 3′ UTR. The luciferase vector was cotransfected with the *Zfp36* expression vector or empty vectors in HEK293 cells, which were treated with ActD to terminate transcription. Total RNAs were prepared at the indicated time points to detect the remaining luciferase mRNA levels after ActD treatment. (*I*) Schematic diagram depicting the location of canonical AU-rich elements (AREs, presented as red triangles) in the *Nppc* 3′ UTR. All data are presented as the mean ± SEM of three independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. In *A*, *B*, and *C*, * indicates a significant difference compared with data at 0 h post-hCG.

are essential for recruiting TTP to the 3′ UTR of target mRNA; therefore, we attempted to determine the potent ARE(s) that direct TTP-induced *Nppc* mRNA degradation. As expected, we found four scattered AUUUA motifs, the canonical ARE sequence, located in the *Nppc* 3′ UTR (Fig. 1*I* and *SI Appendix*, Fig. S4*A*). We then constructed a range of luciferase reporters that contained different mutant ARE motifs (Fig. 2*A*); however, none of the detected canonical ARE motifs were essential for mediating TTP-induced repression of any reporters (Fig. 2*B*). To exclude possible synergistic or additive effects of these canonical ARE motifs, we further constructed a reporter containing the 3′ UTR in which all putative ARE motifs were mutated (Fig. 2*A*). Unexpectedly, in line with the results of each single mutation, the tetrad mutation also led to a significant decrease in luciferase activity (Fig. 2*B*), indicating that the reporter still retains significant AU richness in the 3′ UTR, and implying that other unidentified motif(s) might underlie the TTP-induced degradation of *Nppc* mRNA. This unexpected observation was reminiscent of the presence of noncanonical, but AU-rich 3′ UTR motifs, also known as non-AUUUA AREs. Previous studies have shown that canonical ARE motifs are dispensable for rapid mRNA degradation; however, noncanonical ARE motifs can functionally mediate the rapid destabilization of target mRNA (36, 37). Thus, we screened the *Nppc* 3′ UTR and characterized multiple non-AUUUA but AU-rich motifs. We hypothesized that the *Nppc* 3′ UTR contained repressive noncanonical AREs that could mediate TTP-induced destabilization of *Nppc* mRNA.

To confirm the presence and location of repressive noncanonical ARE motifs, we next constructed a range of luciferase reporters that contained different truncated fragments from the proximal to distal portions of the *Nppc* 3′ UTR (Fig. 2*C*). Similar to the results using the full-length *Nppc* 3′ UTR, overexpression of TTP significantly decreased the luciferase activity of the reporter constructs containing longer proximal regions (1 to 365, 1 to 275), even though the truncated region contained canonical ARE sites (Fig. 2*D*). However, when shorter fragments (1 to 160, 1 to 97) were retained, the luciferase activity of these reporters were not repressed by TTP overexpression, which was comparable to the full-length *Nppc* 3′ UTR deletion reporter construct (Fig. 2*D*). This result suggested that the uncharacterized repressive motifs were located in the 160 to 275 region of the *Nppc* 3′ UTR. Additionally, we observed an increased trend of luciferase activity when distal fragments (downstream from 275) were deleted (Fig. 2*D*), implying that weak repressive AU-rich motifs were located in this region.

Next, we performed a more detailed analysis of the 160 to 275 region of the *Nppc* 3′ UTR and confirmed the location of the noncanonical repressive ARE, which was characterized by UU/UA dinucleotide clusters (231 to 257) (Fig. 2*E*). More importantly, we provided direct evidence that this non-AUUUA ARE motif could functionally mediate TTP-induced destabilization of target mRNA (Fig. 2*F*). Interestingly, by screening the *Nppc* 3′ UTR of different mammalian species, we found that the UU/UA dinucleotide clusters are highly conserved (*SI Appendix*, Fig. S4*B*). Collectively, these
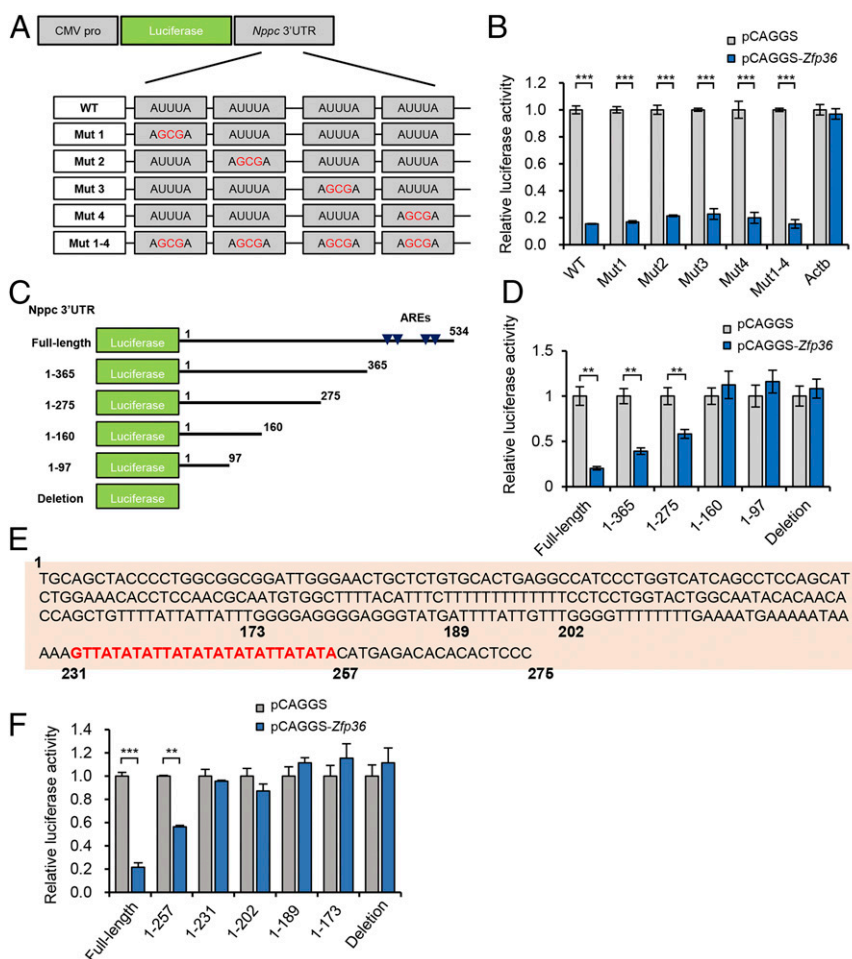
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Fig. 2. TTP targets the noncanonical ARE motifs in the *Nppc* 3′ UTR. (*A*) Schematic diagram of the luciferase reporters that contained single or all mutant canonical ARE motifs in the *Nppc* 3′ UTR. (*B*) Relative luciferase activity of luciferase reporters containing different mutant ARE motifs, which were cotransfected with the *Zfp36* overexpression vector (pCAGGS-Zfp36) or empty vector (pCAGGS) in HEK293 cells. The reporter containing the *Actb* 3′ UTR was detected as a nontargeted control. (*C*) Schematic diagram of the luciferase reporters that contained different truncated regions from the proximal to distal portions of the *Nppc* 3′ UTR. Canonical ARE sites located in the *Nppc* 3′ UTR are indicated by blue arrows. (*D*) Relative luciferase activity of luciferase reporters containing different *Nppc* 3′ UTR truncations, which were cotransfected with the *Zfp36* overexpression vector or empty vectors in HEK293 cells. The reporters that contained full-length or deleted *Nppc* 3′ UTRs were used as positive or negative control, respectively. (*E*) The sequence of the *Nppc* 3′ UTR containing putative noncanonical ARE motifs (highlighted in red). (*F*) Relative luciferase activity of luciferase reporters containing further truncated *Nppc* 3′ UTR fragments. All data are presented as the mean ± SEM of at least three independent experiments. **$P < 0.01$, ***$P < 0.001$.

observations showed that TTP can bind to the *Nppc* 3′ UTR via the noncanonical non-AUUUA ARE motif to destabilize its target mRNA.

Nppc mRNA in Preovulatory MGCs Is Degraded by Up-Regulated TTP.
Having established the correlation between TTP and the noncanonical ARE motif in the *Nppc* 3′ UTR, we next aimed to test whether TTP can regulate *Nppc* expression in preovulatory MGCs. We used a well-established in vitro model using MGCs isolated from preovulatory follicles, which maintain high levels of *Nppc* mRNA via supplementation with estrogen (18, 38). Given that LH failed to decrease *Nppc* mRNA in vitro because of the dilution of LH-induced epidermal growth factor (EGF)-like peptides in the culture medium (18), we recapitulated the LH-induced rapid decrease of *Nppc* mRNA by modulating EGFR signaling, as reported in previous studies (18, 39). In addition, we found that the administration of the EGFR kinase inhibitor, AG1478, completely blocked the hCG-induced rapid up-regulation of TTP (Fig. 3*A*), implying that the activation and function of TTP largely depends on LH/hCG-activated EGFR signaling. Moreover, our results showed that *Zfp36* was rapidly and transiently up-regulated and

that *Nppc* mRNA was decreased in cultured mouse MGCs in response to EGF supplementation (Fig. 3*B*), thus recapitulating the LH/hCG-induced changes in ovaries and MGCs (Fig. 1 *A* and *B* and *SI Appendix*, Fig. S1 *A and B*). These findings support the feasibility of the model to study the role of TTP in controlling endogenous *Nppc* expression. Furthermore, the in vitro model has the unique advantages of MGC-specific genetic manipulation of *Zfp36* and efficient transcriptional termination to assess mRNA stability.

Using the in vitro model, we next determined if TTP up-regulation was essential and sufficient to decrease *Nppc* mRNA levels via loss- and gain-of-function studies. As expected, when the expression of *Zfp36*/TTP was reduced to basal level via small interfering (siRNA)-mediated knockdown (Fig. 3*C* and *SI Appendix*, Fig. S5*A*), EGF supplementation failed to induce the rapid decrease in *Nppc* mRNA but maintained the steady-state expression levels in preovulatory MGCs, which contrasted with the groups transfected with the blank-control and negative-control siRNAs (Fig. 3*C*). In contrast, over-expression of TTP was sufficient to maintain *Nppc* mRNA at low levels in preovulatory MGCs (Fig. 3*D* and *SI Appendix*, Fig. S5*B*). This result was consistent with the observation from the in vivo
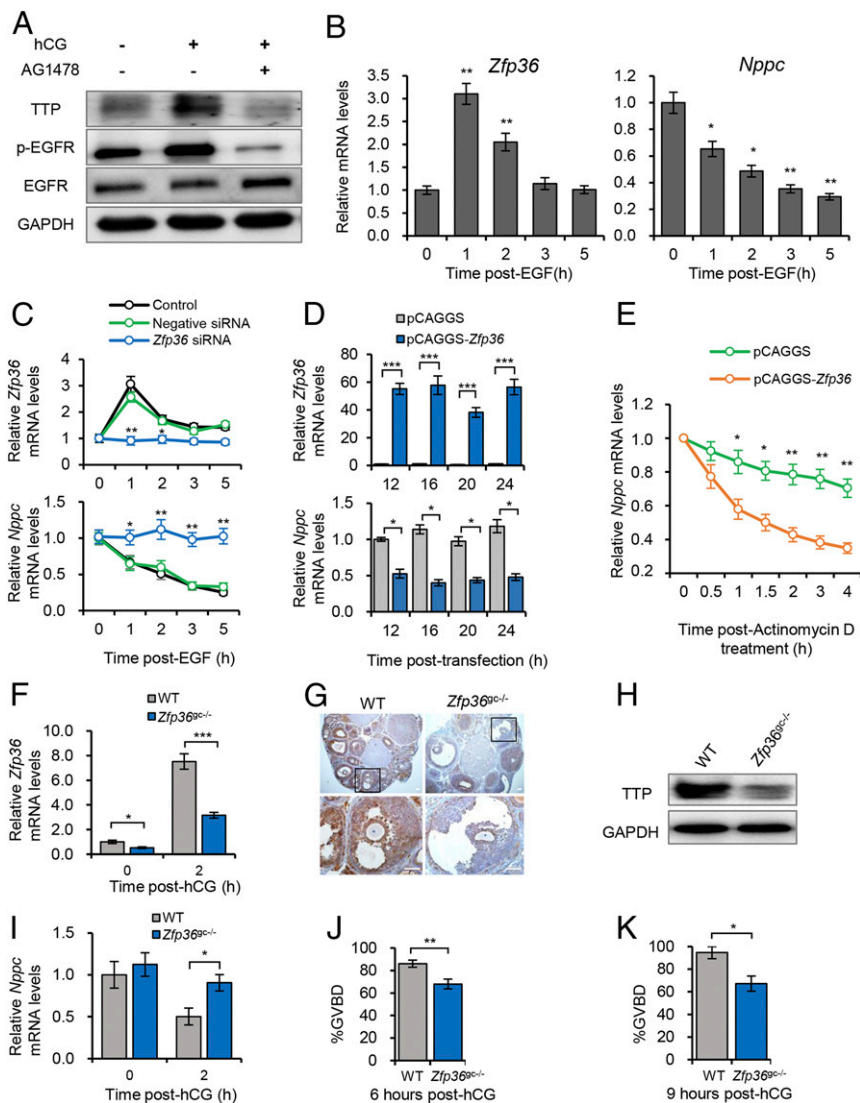
Fig. 3. TTP degrades endogenous *Nppc* mRNA in preovulatory MGCs. (*A*) Western blotting analysis for TTP, as well as phosphorylated and total EGFR, in preovulatory MGCs in response to administration of hCG and AG1478, a specific EGFR inhibitor. MGCs were isolated from in vitro cultured preovulatory follicles. (*B*) Expression dynamics of *Nppc* and *Zfp36* in in vitro cultured MGCs in response to EGF administration. * indicates significant difference compared with data at 0 h post-hCG. (*C*) Effect of *Zfp36* knockdown on the EGF-induced decrease in *Nppc* mRNA in in vitro cultured MGCs. Total RNAs were prepared at the indicated time points after EGF administration to detect *Zfp36* (*Upper*) and *Nppc* (*Lower*) mRNA levels using RT-qPCR. (*D*) Effect of *Zfp36* overexpression on the *Nppc* mRNA levels in in vitro cultured MGCs. *Nppc* expression was maintained at a high level before overexpressing *Zfp36*. Total RNAs were prepared at the indicated time points after transfection to detect *Zfp36* (*Upper*) and *Nppc* (*Lower*) mRNA levels. (*E*) Measurement of the stability of *Nppc* mRNA in MGCs following transfection with the *Zfp36* overexpression vector (pCAGGS-*Zfp36*) or empty vector. Total RNAs were prepared at the indicated time points to detect the remaining *Nppc* mRNA levels after ActD treatment. (*F*–*H*) The detection of *Zfp36* knockout efficiency in MGCs at 2 h post-hCG, at which point *Zfp36* should be fully activated in response to hCG administration. The expression levels of *Zfp36*/TTP in MGCs were detected at 0 h and 2 h post-hCG using RT-qPCR (*F*), immunohistochemistry (*G*), and Western blotting (*H*). (*G, Lower*) Higher magnification of boxed regions. (Scale bar, 100 μm.) (*I*) Effects of MGC-specific depletion of *Zfp36* on *Nppc* mRNA levels at 0 h and 2 h post-hCG. (*J* and *K*) Evaluation of oocyte meiotic resumption scored as GVBD percentage at 6 h (*J*) and 9 h (*K*) post-hCG. Representative images show the morphology of oocytes isolated from preovulatory follicles of wild-type (WT) and *Zfp36*[gc−/−] mice (*Right*), five to eight mice were used at each time point. (Scale bar, 100 μm.) Data show the means ± SEMs of three independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

experiment that down-regulated *Zfp36* was associated with failure to decrease the *Nppc* mRNA level (Fig. 4*C*). Next, we attempted to confirm that TTP-induced rapid decrease in *Nppc* mRNA is dependent on mRNA destabilization. We found that *Nppc* mRNA maintained a relatively stable level until 4 h after ActD treatment; however, *Zfp36* overexpression significantly accelerated the destabilization of *Nppc* mRNA, similar to that of the targeted control *Tnf* mRNA (Fig. 3*E* and *SI Appendix,* Fig. S5*C*). By contrast, accelerated destabilization of nontargeted control *Actb* mRNA was not observed following *Zfp36* overexpression (*SI Appendix,* Fig. S5*D*).

Using cultured bovine MGCs as the model, our results also suggest that the physiological role of EGF-induced TTP in degrading *NPPC* mRNA might be common to different species (*SI Appendix,* Fig. S5 *E–I*).

Finally, to determine the physiological consequences of TTP deficiency in vivo, we crossed *Zfp36*[flox/flox] mice and *Fshr-Cre* mice to knock out *Zfp36* specifically in MGCs. We detected that in the resulting *Zfp36*[gc−/−] mice, *Zfp36* expression was significantly decreased in preovulatory MGCs before and after hCG administration (Fig. 3*F*). The reduction in TTP protein levels in MGCs was
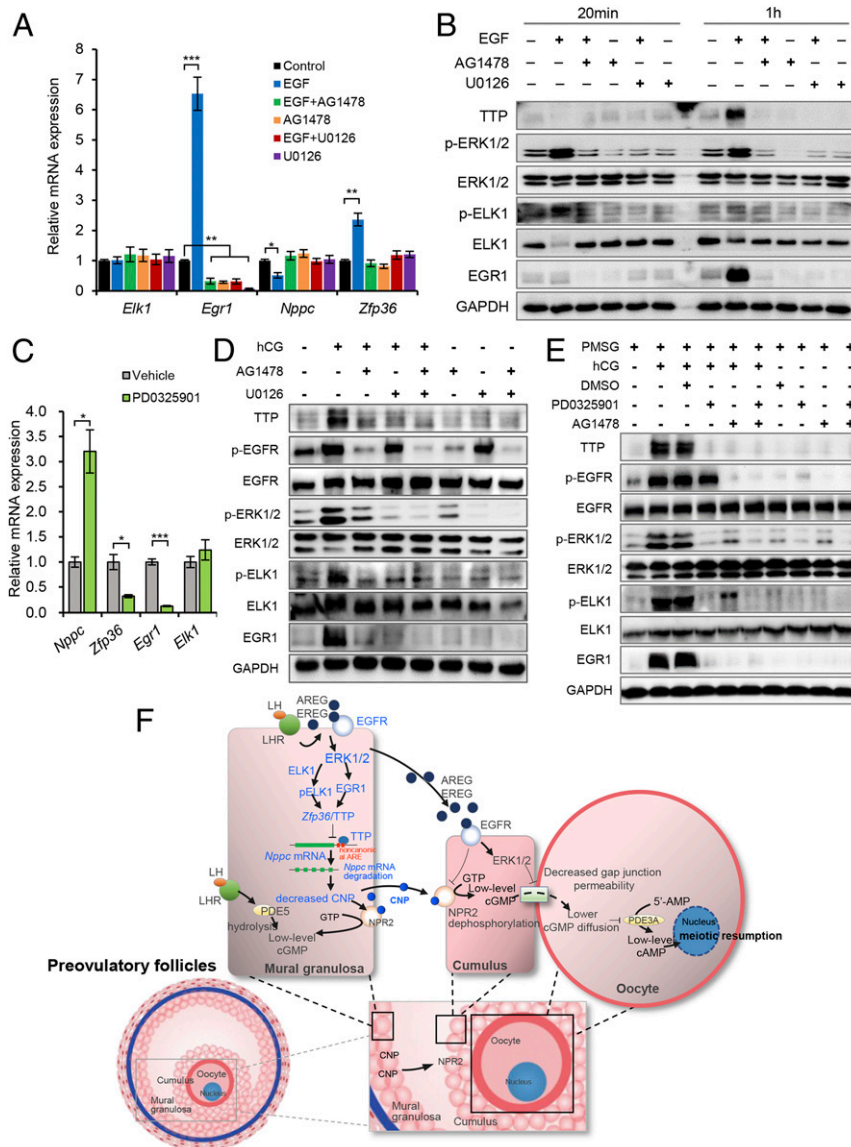
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https://doi.org/10.1073/pnas.2018345118

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Fig. 4. LH/hCG activates *Zfp36*/TTP through the EGFR-ERK1/2–dependent pathway. (*A*) Effect of the EGFR-ERK1/2 pathway on the expression of *Zfp36* and its associated transcription factors. mRNA expression levels of *Zfp36*, *Nppc*, *Elk1*, and *Egr1* in in vitro cultured MGCs that were treated with either EGF or AG1478 (a specific EGFR inhibitor), U0126 (a specific ERK inhibitor), or their various combinations were detected. AG1478 or U0126 was administered 1 h before EGF supplementation. (*B*) Western blotting analysis for TTP, EGR1, and phosphorylated and total ERK1/2 or ELK1 in in vitro cultured MGCs that were treated with either EGF, AG1478, U0126, or their various combinations for 20 min and 1 h. (*C*) Expression levels of *Zfp36*, *Nppc*, *Elk1*, and *Egr1* mRNA in isolated preovulatory MGCs from mice that were intraperitoneally administrated with hCG, with or without PD0325901 (a specific ERK inhibitor). MGCs were collected at 3 h after hCG administration, and PD0325901 was injected at 4 h before hCG. (*D*) Western blotting analysis for TTP, EGR1, and phosphorylated and total EGFR, ERK1/2, or ELK1 in MGCs from in vitro cultured preovulatory follicles that were treated with either hCG, AG1478, U0126, or their various combinations. (*E*) Western blotting analysis for TTP, EGR1, and phosphorylated and total EGFR, ERK1/2, or ELK1 in isolated preovulatory MGCs from mice that were injected intraperitoneally with either PD0325901, AG1478, or their various combinations at 4 h before hCG administration. All data are presented as the mean ± SEM of at least three independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. (*F*) Working model of the signaling pathways that regulate the CNP-NPR2-cGMP signaling cascade and meiotic resumption in preovulatory follicles. Pathways reported previously and those identified in the present study are indicated in black and blue, respectively.

further confirmed by immunohistochemical and Western blotting detection at 2 h post-hCG (Fig. 3 *G* and *H*), at which point the high-level expression of *Zfp36* should appear in preovulatory follicles (Fig. 1*B* and *SI Appendix*, Fig. S1*B*). Incomplete up-regulation of TTP/*Zfp36*, in turn, impaired the hCG-induced rapid decrease in *Nppc* mRNA (Fig. 3*I*). When oocyte meiotic resumption, scored as percent germinal vesicle breakdown (GVBD), was evaluated, *Zfp36*^gc−/− mice showed impaired meiotic resumption compared with that in the wild-type controls (Fig. 3 *J* and *K*). These findings were also supported by results of lentivirus-mediated short hairpin RNA (shRNA) interference of *Zfp36* in preovulatory

follicles (*SI Appendix*, Fig. S6 *A–F*). Collectively, our results from in vitro and in vivo models demonstrated that TTP-dependent mRNA degradation underlies the rapid decrease in *Nppc* mRNA in preovulatory follicles, which plays a functional role in fine-tuning the progression of oocyte meiosis.

LH/hCG Activates *Zfp36* through the EGFR-ERK1/2–Dependent Pathway.
Having confirmed the functional role of TTP-mediated mRNA degradation of *Nppc* mRNA in preovulatory MGCs, we next attempted to understand the mechanism underlying the transient up-regulation of *Zfp36* in response to the LH/hCG surge. Multiple

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The mRNA-destabilizing protein Tristetraprolin targets "meiosis arrester" *Nppc* mRNA in
mammalian preovulatory follicles

molecular and cellular evidence indicated that *Zfp36* transcription is induced by E26 transformation-specific (ETS) transcription factor ELK1 (ELK-1) and early growth response 1 (EGR-1) in the EGFR-ERK1/2–dependent pathway in somatic cells (40–43). Given that LH-activated EGFR-ERK1/2 signaling in preovulatory follicles is essential to initiate meiotic resumption (22, 44), we hypothesized that the rapid induction of *Zfp36*/TTP in response to LH/hCG might depend on the EGFR-ERK1/2 pathway. Therefore, we examined the hypothesis using both in vitro and in vivo models. In in vitro cultured MGCs, accompanied by the rapidly increased *Zfp36* and decreased *Nppc* mRNA levels, we observed that the transcriptional activation of *Egr1*, but not *Elk1*, responded rapidly to EGF stimulation through the EGFR-ERK1/2–dependent pathway (Fig. 4A). Furthermore, Western blotting and immunofluorescence assays showed that ERK1/2, as well as transcription factor ELK1, could be phosphorylated (activated) in response to EGF stimulation within 20 min (Fig. 4B and *SI Appendix*, Fig. S7 *A and B*). Subsequently, a marked increase in EGR1 was observed after 1 h (Fig. 4B and *SI Appendix*, Fig. S7C), along with significantly increased TTP levels (Fig. 4B). All these changes in response to EGFR signaling activation could be attenuated by AG1478 (an EGFR signaling-specific inhibitor) or U0126 (an ERK1/2-specific inhibitor) (Fig. 4 *A* and *B*). Moreover, to further confirm the dependence of TTP induction on the ERK1/2 pathway, mice were intraperitoneally injected with the ERK1/2 inhibitor 4 h before hCG administration. Consistent with the results from the in vitro model, in vivo inhibition of the ERK1/2 pathway significantly blocked the induction of *Egr1* and *Zfp36* expression, and the decrease in *Nppc* mRNA levels (Fig. 4C). Thus, the results of in vitro and in vivo assays indicated that the ERK1/2 pathway is essential for rapidly activating the transcriptional induction system of *Zfp36*.

Given that EGFR is not the only mediator of LH/hCG-induced ERK1/2 activation (45–47), we next examined if EGFR was indispensable in mediating the LH/hCG-induced activation of the ERK1/2 pathway and downstream transcriptional induction system of *Zfp36*. To this end, isolated mouse preovulatory follicles were cultured and treated with hCG, which resulted in the hCG-induced activation/expression of the ERK1/2 pathway, as well as TTP (Fig. 4D). Additionally, the changes in transcription factor levels could be completely reversed by blocking EGFR signaling (Fig. 4D). ERK inhibition did not attenuate EGFR phosphorylation/activation (Fig. 4D). Notably, EGFR inhibition and ERK inhibition, alone or in combination, showed a similar effect of blocking hCG-induced TTP expression (Fig. 4D), suggesting that the ERK1/2 pathway acts downstream of EGFR signaling. These findings were further confirmed using in vivo models (Fig. 4E). Collectively, these results demonstrated that the preovulatory LH surge induces transient expression of *Zfp36*/TTP and its transcriptional induction system via the EGFR-ERK1/2 signaling pathway.

Discussion

The progression of gonadotropin-controlled oocyte meiosis is tightly regulated by the activity of the CNP-NPR2-cGMP signaling cascade. Before the LH surge, CNP-NPR2-cGMP signaling maintains the active state. The "arrester," CNP, is primarily synthesized by MGCs and binds to NPR2 throughout the follicle to stimulate cGMP production, thus maintaining meiotic arrest. Following the LH surge, CNP-NPR2-cGMP signaling inhibition occurs consecutively on multiple levels; however, it remains unclear what mechanism maintains the inhibition of the cascade, achieving sustained cGMP at low levels before and during meiotic resumption. Earlier studies proposed that the LH-induced rapid dephosphorylation (inactivation) of NPR2 guanylyl cyclase might contribute to CNP-NPR2-cGMP signaling inhibition, thus causing the rapid decrease in cGMP. However, further studies have indicated that the NPR2 dephosphorylation-dependent rapid decrease in cGMP (13, 14) is dispensable for LH-induced meiotic resumption because the mouse model that prevents NPR2 dephosphorylation (inactivation)

only attenuated part of the cGMP decrease, which resulted in delayed, but not failed, meiotic resumption (16). Similarly, LH-induced phosphodiesterase 5 (PDE5), which directly hydrolyzes cGMP, only partially contributes to the rapid cGMP decrease and does not affect the timing of meiotic resumption. More importantly, mice carrying both active NPR2 and mutated PDE5 failed to completely block cGMP decrease (12). Therefore, these results are reminiscent of upstream changes to the signaling cascade (i.e., the LH/hCG-induced decrease in follicular ligand CNP and its encoding mRNA *Nppc*), which was documented as an important fail-safe mechanism that may ensure LH-induced CNP-NPR2-cGMP signaling inhibition and meiotic resumption (22).

Our data further emphasized the importance of ovarian follicular somatic cells in regulating cGMP homeostasis. To maintain cGMP at high levels and sustain meiotic arrest, somatic cells not only produce the ligand (CNP) that activates its guanylyl cyclase (NPR2) (11) but also provide guanylyl substrate for de novo synthesis of cGMP (48). After the LH surge, cGMP begins to decrease rapidly in somatic cells via the synergistic effect of decreased activity and affinity of NPR2 guanylyl cyclase (14–16), direct hydrolysis of cGMP (12), and decreased concentrations of CNP (17–19). Functional redundancy also suggests that the timing of meiotic resumption and ovulation is critical, and multiple pathways might have evolved as a fail-safe system to ensure LH-induced meiotic resumption. Compared with the rapid decrease of NPR2 activity and activated hydrolysis of cGMP that occurs within 30 min, the decrease of CNP is a relatively slow event (50% decrease within ~2 h and further decrease until the nuclear envelope is completely broken down) but is concurrent with the progression of meiotic resumption (14). Thus, the decrease of CNP/*Nppc* in the preovulatory follicle was thought to potentially contribute to driving meiotic resumption, and the mechanism underlying the decrease was considered as an important and long-standing unanswered question (14, 22). Our study supported the idea that the delayed decrease in *Nppc* mRNA could impair the progression of meiotic resumption. These results, together with previous works that focused on NPR2 inactivation and cGMP hydrolysis (12, 14, 16), allow us to presume that NPR2 inactivation and cGMP hydrolysis may preferentially contribute to the rapid initiation of meiotic resumption, while *Nppc* mRNA decline is primarily responsible for the later meiotic progression.

In the present study, we revealed that the preovulatory LH surge can stimulate *Zfp36* expression via activation of ELK-1 and EGR-1 transcription factors through the EGFR-ERK1/2 signaling pathway. Up-regulated TTP subsequently targets noncanonical ARE motifs in the 3′ UTR of the *Nppc* mRNA, which induces its rapid degradation (Fig. 4F). The turnover of CNP is rapid (24), and the LH-induced decrease in the CNP peptide is independent of the natriuretic peptide clearance receptor NPR3 (18); therefore, the rapid degradation of *Nppc* mRNA would lead to a decrease in CNP peptide levels, thus maintaining CNP-NPR2-cGMP signaling inhibition during meiotic resumption.

We provided the functional link between TTP-mediated mRNA degradation and oocyte meiotic progression. The involvement of mRNA degradation-mediated posttranscriptional mechanism in regulating CNP-NPR2-cGMP signaling has never been reported. Furthermore, our results highlight the role of the rare noncanonical AU-rich element in this process. Among the ARE-binding proteins that regulate posttranscriptional mechanisms, TTP has the most specific recognition and binding ARE sequences (i.e., canonical AUUUA motifs) (26, 27). However, non-AUUUA, but AU-rich motifs, can also destabilize target mRNA (36, 37, 49). The UU/UA dinucleotide clusters observed in the *Nppc* 3′ UTR, although generally rare in vertebrate genomes, were shown to correlate positively with TTP-induced mRNA destabilization (49). Our results, together with those of previous studies, supported the idea that noncanonical ARE motifs efficiently mediate the mRNA-destabilizing function.

The present study identified that TTP-induced mRNA degradation, a global posttranscriptional regulatory mechanism, acts as a

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regulatory component of orchestrating the signals for oocyte meiotic maturation. We report that *Nppc* mRNA is the functional target of TTP, via rare noncanonical ARE motifs. This finding might shed light on the mechanism of CNP-dependent cGMP homeostasis in regulating other physiological processes, considering the significant regulatory role of CNP-NPR2-cGMP signaling in the development and function of the cardiovascular, skeletal, and nervous systems.

Materials and Methods

Animal Studies and Ethical Approval. All female ICR mice used in this study were from Beijing Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). *Fshr-Cre* mice and *loxP*-flanked *Zfp36* mice (*Zfp36*^flox/flox) were gifts from Dr. Louis Dubeau (University of Southern California, Los Angeles, CA) and Dr. Perry J. Blackshear (National Institute of Environmental Health Sciences, NIH), respectively. All mice were maintained in a climate-controlled room on a 12 h light/darkness cycle and allowed food and water ad libitum. The China Agricultural University Institutional Animal Care and Use Committee approved

this study, which was performed in accordance with the committee's guidelines. All efforts were made to minimize animal suffering.

Cell culture, isolation, and culture of follicles, Western blotting, immunofluorescence and histologic analysis, RIP are described in *SI Appendix, Materials and Methods*.

Data Availability. All study data are included in the article and/or *SI Appendix*.

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