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RESEARCH ARTICLE

The translation regulator Zar1l controls timing of meiosis in *Xenopus* oocytes

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ABSTRACT

Oocyte maturation and early embryo development occur in vertebrates in the near absence of transcription. Thus, sexual reproduction of vertebrates critically depends on the timely translation of mRNAs already stockpiled in the oocyte. Yet how translational activation of specific mRNAs is temporally coordinated is still incompletely understood. Here, we elucidate the function of Zar1l, a yet uncharacterized member of the Zar RNA-binding protein family, in *Xenopus* oocytes. Employing TRIM-Away, we demonstrate that loss of Zar1l accelerates hormone-induced meiotic resumption of *Xenopus* oocytes due to premature accumulation of the M-phase-promoting kinase cMos. We show that Zar1l is a constituent of a large ribonucleoparticle containing the translation repressor 4E-T and the central polyadenylation regulator CPEB1, and that it binds directly to the cMos mRNA. Partial, hormone-induced degradation of Zar1l liberates 4E-T from CPEB1, which weakens translational repression of mRNAs encoding cMos and likely additional M-phase-promoting factors. Thus, our study provides fundamental insights into the mechanisms that ensure temporally regulated translation of key cell cycle regulators during oocyte maturation, which is essential for sexual reproductivity.

KEY WORDS: Zar1l, Zar proteins, Meiosis, *Xenopus laevis*, Translation, RNP

INTRODUCTION

Haploid gamete cells of sexually reproducing species are formed in a process termed meiosis, which is a highly specialized type of cell cycle where DNA replication in pre-meiotic S-phase is followed by two consecutive rounds of chromosome segregation and cell division (MI and MII) without intervening DNA replication (Jessus et al., 2020). In vertebrates, immature oocytes are arrested in a G2-like state during MI (prophase I arrest, referred to as stage VI in *Xenopus laevis*), which is characterized by low activity of cyclin-dependent kinase 1 (Cdk1). Hormonal stimulation [e.g. progesterone (PG) in *Xenopus laevis*] triggers Cdk1 activation, resulting in nuclear envelope breakdown [termed germinal vesicle breakdown (GVBD)], completion of MI and transition into MII (Jessus et al., 2020). This process, termed meiotic maturation, is accompanied by a plethora of coordinated changes in proteome composition and associated post-translational modifications. Timely activation of Cdk1 in *Xenopus* oocytes requires synthesis of its co-activators RINGO/Speedy and cyclin B1, as well as of the kinase cMos (Meneau et al., 2020). Notably, meiotic maturation happens in the near absence of transcription; therefore, the translation of specific proteins from stockpiled mRNAs at distinct cell cycle stages has to be tightly controlled (Christou-Kent et al., 2020).

Cap-dependent translation is initiated at the 5′end of the mRNA by binding of the eIF4F and eIF3 complexes, which in concert mediate subsequent recruitment of the small ribosomal subunit and additional translation initiation factors (Aitken and Lorsch, 2012). The efficiency of ribosome recruitment and unwinding of RNA secondary structures can furthermore be dynamically stimulated by the non-essential initiation factor eIF4B (Özeş et al., 2011; Walker et al., 2013). The eIF4F complex consists of the cap-binding protein eIF4E, the helicase eIF4A and the scaffold protein eIF4G, which, according to the 'closed-loop' model of translation initiation, binds to polyadenylate-binding proteins (PABP) associated with the polyA-tail (Wells et al., 1998). Thus, by physically linking the 5′cap with the 3′polyA-tail, this conformation enhances productive translation of the mRNA (Jackson et al., 2010). Additional regulatory proteins present in these ribonucleoparticles (RNP) can modulate this network of protein-protein and protein-RNA interactions to control the translation efficiency of specific mRNAs (Christou-Kent et al., 2020). During meiotic maturation, dynamic remodeling of RNPs induces changes in the length of the polyA-tail of many mRNAs, which directly correlates with translation efficiency (Weill et al., 2012; Yang et al., 2020). Lengthening of the polyA-tail during meiotic maturation is mainly controlled by two cis-acting elements in the 3′UTR of mRNAs. One is the polyadenylation signal (PAS), which usually occurs around 15 nucleotides (nt, in humans) or 24 nt (in *Xenopus* oocytes) upstream of the processed 3′end of the mRNA and is recognized by the multi-subunit cleavage and polyadenylation specificity factor (CPSF) that in the cytoplasm can recruit the polyA-polymerase Gld2 (Barnard et al., 2004; Clerici et al., 2018; Wu and Bartel, 2017; Yang et al., 2020). The other element is the cytoplasmic polyadenylation element (CPE), which is recognized by the cytoplasmic polyadenylation element binding protein 1 (CPEB1) (Hake and Richter, 1994). In addition to the PAS and CPE, other cis-acting elements, such as the Musashi-binding element (MBE), the Pumilio-binding element (PBE) and the translational control sequence (TCS), have been described to contribute to the control of polyadenylation and translation of mRNAs during meiotic maturation. The MBE recruits the trans-acting factor Musashi1 (Msi1) and it has been shown that this modifies the use of specific CPE elements by CPEB1 in the 3′UTR of the cMos mRNA, a process that is required for timely cMos expression during meiotic maturation in *Xenopus laevis* (Charlesworth et al., 2006; Cragle et al., 2019; Weill et al., 2017). The PBE is bound by the Pumilio

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proteins Pum1 and Pum2, which leads to repression of the associated mRNA, e.g. of the Cdk1 activators cyclin B1 and RINGO/Speedy (Ota et al., 2011; Padmanabhan and Richter, 2006; Takei et al., 2020). The TCS was initially identified as a novel cis-acting element controlling the precise timing of Wee1 and Pcm-1 translation during meiosis in *Xenopus laevis*; later it was shown that the TCS is bound by the trans-acting factors Zygote arrest 1 (Zar1) and Zygote arrest 2 (referred to as Zar2 here, but also described as Zar1-like in the literature) (Charlesworth et al., 2012; Wang et al., 2008; Yamamoto et al., 2013). Reportedly, the cMos mRNA also carries a TCS sequence that can be bound by both proteins, although the relevance of this for timely cMos translation during *Xenopus* meiosis is unclear (Charlesworth et al., 2012; Yamamoto et al., 2013). Zar proteins have been implicated in regulation of translation during female meiosis in several vertebrate species and their absence has been correlated with a variety of meiotic defects that ultimately result in infertility (Miao et al., 2017; Michailidis et al., 2010; Rong et al., 2019; Wu and Fan, 2022; Yamamoto et al., 2013). The TCS of the Wee1 mRNA has been shown to confer repression of translation in immature *Xenopus* oocytes and to activate polyadenylation and translation during hormone-induced meiotic resumption (Wang et al., 2008). Accordingly, both Zar1 and Zar2 repress translation in reporter assays in immature *Xenopus* oocytes (Yamamoto et al., 2013). The zebrafish Zar1 protein has been shown to interact with 4E-T, CPEB1 and ePAB, which have all been described as part of a repressive RNP in early *Xenopus* oocytes (Miao et al., 2017; Minshall et al., 2007). In mouse, Zar1 and Zar2 are required for faithful accumulation of mRNAs during early oogenesis and, somewhat surprisingly, for timely polyadenylation rather than repression of mRNAs (Rong et al., 2019). Here, we dissect the function of Zar1l, a hitherto uncharacterized member of the Zar protein family, in *Xenopus laevis* oocytes. Using a combination of pull-down, depletion and mass spectrometry experiments, we find that Zar1l is part of a repressive RNP containing 4E-T and other known associated factors. We further reveal that depletion of Zar1l results in premature activation of Cdk1 during hormone-induced meiotic maturation and provide evidence that this effect depends on deregulated control of translation via the cMos 3′UTR.

RESULTS

Zar1l is distinct from Zar1 and Zar2, and is expressed during meiotic maturation in *Xenopus laevis*

The Zar protein family has been implicated in translational regulation during female meiosis in a variety of vertebrate species and so far two members, Zar1 and Zar2 (also known as Zar1-like), have been described (Sangiorgio et al., 2008; Wu et al., 2003; Yamamoto et al., 2013). Interestingly, a third, and so far functionally uncharacterized, member of the Zar protein family has been identified in *Xenopus laevis*. This protein, termed Zar1l (Zar1l.L/XP_018103300.1 and Zar1l.S/XP_018105973.1), cannot be assigned unequivocally to the Zar1 or Zar2 subfamily because it carries characteristic amino acids of both groups (Fig. 1A) (Yamamoto et al., 2013). Likewise, calculating total amino acid identity between Zar1l and Zar1 or Zar2 revealed similar values, both when the full-length proteins were analyzed and when the highly conserved C-terminal domain was omitted (Fig. 1B, Fig. S1A).

To better understand the function of Zar1l during female meiosis, we first raised antibodies (Ab) against Zar1, Zar1l and Zar2, and confirmed their specificity by testing them against *in vitro* translated (IVT) Flag-tagged S- and L-versions of all known *Xenopus* Zar proteins (Fig. 1C). Next, we analyzed the expression pattern of Zar1l during meiotic maturation in *Xenopus* oocytes. To this end, we treated *Xenopus* stage VI oocytes with PG to trigger meiotic resumption, which was confirmed by loss of inhibitory Cdk1 phosphorylation (ppCdk1) (Fig. 1D). We observed that Zar1l is expressed in stage VI oocytes and its levels clearly decreased after PG addition, with much of the degradation happening within 1 h (Fig. 1E). Of note, this degradation was incomplete and ceased 2 h after PG addition (Fig. 1D). This degradation during meiotic maturation closely resembles the previously reported pattern of *Xenopus* Zar2 (Charlesworth et al., 2012). The cytostatic factor XErp1 accumulates during late MI and secures the arrest of mature oocytes at metaphase of MII (Schmidt et al., 2005). The remaining pool of Zar1l was not further destabilized during this developmental period.

Zar1l depletion deregulates cMos expression and meiotic maturation

Next, we assessed whether Zar1l, like Zar1 and Zar2 (Miao et al., 2017; Rong et al., 2019), controls mRNA translation in oocytes. To test this, we depleted Zar1l from immature oocytes using the TRIM-Away approach (Clift et al., 2017). This approach exploits the E3 ligase and cytosolic Ab receptor TRIM21 to target endogenous proteins recognized by an Ab for proteasomal degradation (Zeng et al., 2021). *Xenopus* stage VI oocytes were injected with mRNA encoding Flag-tagged TRIM21 E3 ligase in combination with $Zar1l^{Ab1}$ antibody. Immunoblot analyses of oocytes 18 h and 42 h after injection revealed that endogenous Zar1l was efficiently depleted in oocytes co-injected with Flag-TRIM21 and $Zar1l^{Ab1}$, but not with control (Ctrl) antibodies (Fig. 2A). Co-injection of $Zar1l^{Ab1}$ with mRNA encoding a C-terminally truncated version of TRIM21 ($TRIM21^{\Delta C}$) that cannot bind the Fc-domain of antibodies (Clift et al., 2017) did not result in Zar1l depletion, confirming that the knockdown requires recruitment of TRIM21 to the antibody. With this tool in hand, we aimed to investigate potential functional consequences of Zar1l depletion by treating these oocytes with PG. Surprisingly, although Zar1l-depleted oocytes did not mature spontaneously in the absence of PG, they proceeded much faster to GVBD upon PG stimulation than did Ctrl-depleted oocytes (Fig. 2B,C). GVBD was determined by the appearance of a characteristic white spot in the animal hemisphere of the *Xenopus* oocyte (Okada et al., 2012). This is in stark contrast to oocytes from Zar1 and Zar2 double knockout mice, which were delayed rather than accelerated in meiotic resumption (Rong et al., 2019). To confirm that the observed phenotype is due to Zar1l depletion, we expressed Zar1l variants with mutations in the $Zar1l^{Ab1}$ targeting epitope in Zar1l-depleted oocytes. Unfortunately, these efforts were not successful. We speculated that Zar1l re-expression was not sufficient to rescue the phenotype because depletion of Zar1l caused the destabilization of other RNP components such as 4E-T (see Fig. 6B), a phenomenon commonly observed when subunits of multi-protein complexes are present in the wrong stoichiometry (Taggart et al., 2020). Alternatively, the introduced mutations interfered with Zar1l function. Nevertheless, we are convinced that the phenotype was specific because TRIM21-mediated depletion of Zar1l using $Zar1l^{Ab2}$, which recognizes an antigen region distinct to the one of $Zar1l^{Ab1}$, resulted in the same phenotype (Fig. 2A-C, Fig. S2A). Notably, prolonged overexpression of Zar1l mimicked the depletion phenotype in that oocytes with increased Zar1l levels resumed meiosis faster upon PG treatment than control oocytes (Fig. S3A). From these data, we concluded that altered Zar1l levels interfere with the physiological function of the RNPs to which Zar1l

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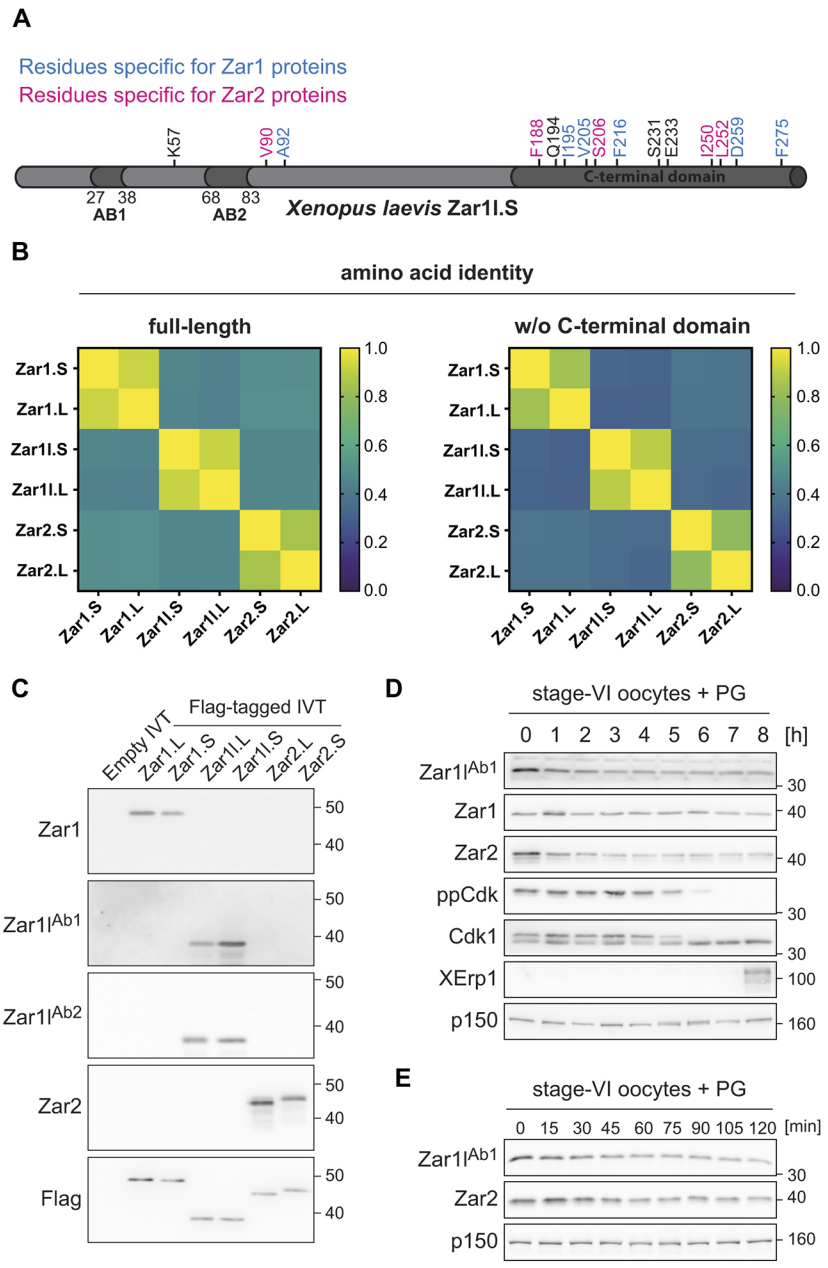

Fig. 1. Zar1l is a novel member of the Zar protein family expressed during *Xenopus* meiotic maturation. (A) Schematic representation of Zar1l.S from *Xenopus laevis*. The conserved C-terminal domain and the two antigens used to raise antibodies 1 (AB1) and 2 (AB2) are indicated in dark gray. According to Yamamoto et al. (2013), the numbered amino acids are characteristic for distinguishing between Zar1 and Zar2 from amphibians to mammals. Blue and magenta indicate identical residues between Zar1l and Zar1, and Zar1l and Zar2, respectively. Zar1l residues that cannot be assigned to either Zar1 or Zar2 are in black. (B) Heat map showing amino acid identity between the L- and S-versions of the *Xenopus laevis* Zar family proteins. Left, between full-length proteins; right, without the conserved C-terminal RNA-binding domain (amino acid Gln179-Ile281 of Zar1l.S). (C) *In vitro* translated (IVT) Flag-tagged L- and S-versions of the Zar family proteins in *Xenopus laevis* were immunoblotted with the indicated antibodies. Empty IVT reaction not expressing a Zar protein served as a control. (D,E) Stage VI oocytes were treated with PG. Samples were taken at the indicated time points and immunoblotted with the indicated antibodies. One representative experiment of three biological replicates is shown.

normally binds and this results in faster meiotic resumption upon PG stimulation.

Intrigued by the fact that oocytes with altered Zar1l levels resume meiosis faster, we aimed to better understand the underlying molecular mechanism. In *Xenopus laevis* oocytes, timely activation of Cdk1 requires a complex pathway of partially redundant and interconnected processes involving translation of the Cdk1 activators cyclin B1 and RINGO/Speedy, as well as of the kinase cMos (Ferby et al., 1999; Haccard and Jessus, 2006; Lenormand et al., 1999). As Zar1 and Zar2 have been shown to be able to bind the 3′UTR of the cMos mRNA (Yamamoto et al., 2013), we speculated that untimely translation of cMos could account for the accelerated meiotic maturation. To test this, we first depleted Zar1l from stage VI oocytes and after PG addition analyzed cMos expression by immunoblot. Interestingly, compared with Ctrl-depleted oocytes, cMos was prematurely expressed in Zar1l-depleted oocytes and this coincided with activation of its downstream effector MAPK, as judged by the appearance of its activating phosphorylation (Fig. 2D). MAPK promotes Cdk1 activation in a dual manner in that it positively acts on the Cdk1-activating phosphatase Cdc25 and negatively on the Cdk1-inhibiting kinase Myt1 via its downstream target p90RSK (Palmer et al., 1998; Wang et al., 2007). In addition, cMos directly inhibits Myt1 independently of the MAPK pathway (Peter et al., 2002). In line with the observed acceleration of meiotic resumption, inhibitory phosphorylation of Cdk1 disappeared earlier in Zar1l-depleted oocytes compared with Ctrl-depleted oocytes (Fig. 2D). Earlier cMos expression and Cdk1 activation was not only observed upon Zar1l depletion, but also upon its prolonged overexpression, which indicates that their common phenotype might have a similar molecular origin (Fig. S3A). If activation of the MAPK pathway by premature expression of cMos accounts for accelerated meiotic resumption of Zar1l-depleted oocytes, inhibition of the MAPK kinase MEK by U0126 should abrogate this effect. In line with the central function of the cMos-MAPK pathway for meiotic resumption (Gross et al., 2000), MEK inhibition resulted in a strong delay of GVBD in Ctrl-depleted

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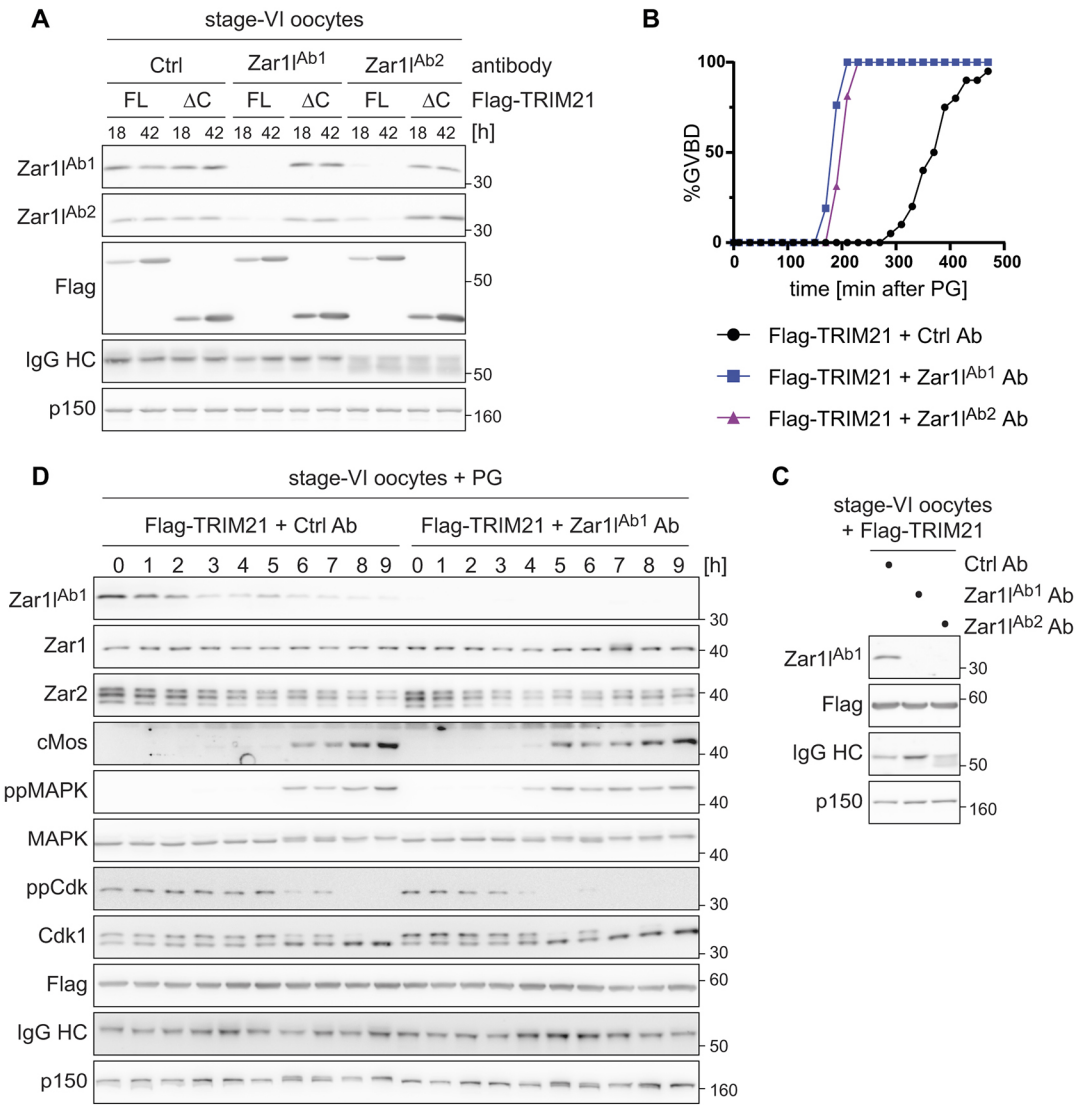

Fig. 2. Depletion of Zar1l accelerates meiotic resumption. (A) Stage VI oocytes were co-injected with the indicated Zar1l or unspecific control (Ctrl) antibodies and with mRNA encoding Flag-tagged full-length (FL) TRIM21 or truncated (ΔC) TRIM21 deficient in antibody binding. Samples were taken at the indicated time points and immunoblotted as indicated. One representative experiment of two biological replicates is shown. (B,C) Stage VI oocytes were co-injected with the indicated Zar1l or unspecific control (Ctrl) antibodies and mRNA encoding Flag-TRIM21. Samples were taken 42 h after injection and immunoblotted with the antibodies indicated in C. In parallel, oocytes (Ctrl, *n*=20; Zar1l^{Ab1}, *n*=21; Zar1l^{Ab2}, *n*=16) were treated with PG and time until GVBD was determined by the appearance of a white spot in the animal hemisphere of the oocyte in B (representative images shown in Fig. S2A). One representative experiment of three biological replicates is shown. (D) Stage VI oocytes were co-injected with Zar1l^{Ab1} or unspecific control (Ctrl) antibodies and mRNA encoding Flag-TRIM21. 42 h after injection, oocytes were treated with PG, samples were taken at the indicated time points and immunoblotted as indicated. One representative experiment of three biological replicates is shown.

oocytes (Fig. S3B). Notably, U0126 treatment similarly slowed down meiotic resumption of Zar1l-depleted oocytes, but these oocytes were still faster than U0126-treated control oocytes. From these data, we concluded that untimely expression of cMos is indeed involved in accelerated GVBD in the absence of Zar1l and that, in addition to the downstream MAPK pathway, cMos itself, e.g. by directly inhibiting Myt1 (Peter et al., 2002), or other prematurely expressed meiotic regulators contribute to faster Cdk1 activation.

Next, we investigated how Zar1l affects the expression timing of cMos. To this end, we first performed immunoprecipitation (IP) experiments to analyze whether Zar1l is part of an RNP that directly binds the cMos mRNA. Immature oocytes were injected with Flag-Zar1l mRNA and expressed Flag-Zar1l was immunoprecipitated followed by the isolation of associated RNA. Of note, for all IP experiments Zar1l was expressed for only 18 h to prevent strong accumulation of Zar1l, which results in accelerated meiotic maturation (see Fig. S3A). Semi-quantitative RT-PCR analyses using primer pairs specific for the 3′UTR of the cMos mRNA revealed a strong enrichment of cMos mRNA in the Flag-Zar1l IP samples compared with the IP from control water-injected oocytes (Fig. 3A,B). Binding of Zar1l to the cMos mRNA was at least in part mediated by a direct interaction between Zar1l and the mRNA, as significantly less cMos mRNA was associated with Flag-Zar1l carrying mutations in the putative RNA-binding domain (2Cys^{-}) (Yamamoto et al., 2013).

Next, we assessed how Zar1l affects the translation efficiency of cMos mRNA. To uncouple translational activation of cMos from its protein stability during meiotic maturation, we created a reporter mRNA consisting of the Flag-eGFP open reading frame (ORF) fused to the complete 3′UTR of the cMos mRNA. As control,

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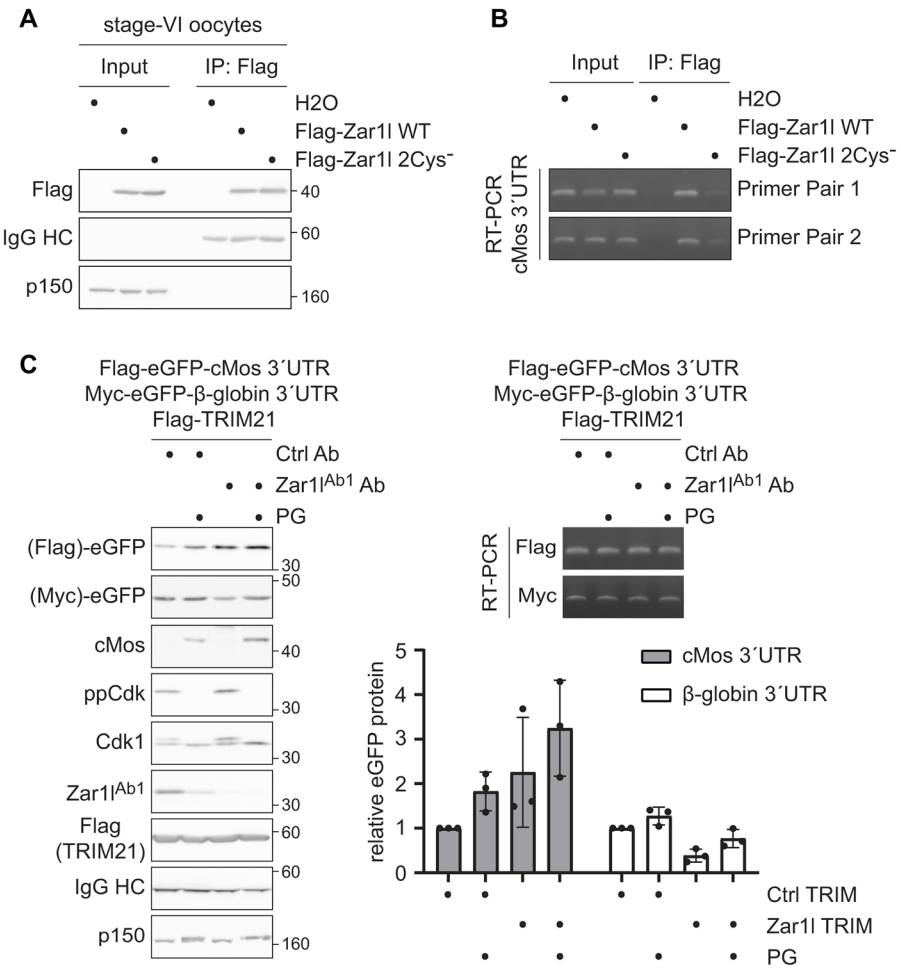

Fig. 3. Zar1l binds the cMos mRNA and deregulates its translation. (A) Stage VI oocytes were injected with mRNA encoding Flag-Zar1l.S that was either wild type (WT) or mutated to alanine at Cys190 and Cys217 (2Cys⁻). After 18 h incubation, oocyte lysates were subjected to anti-Flag immunoprecipitation (IP) and samples were immunoblotted as indicated. (B) RNA was isolated from samples in A and reverse transcribed to cDNA. RT-PCR with two primer pairs specific for the cMos.L 3′UTR was performed to semi-quantitatively determine cMos mRNA levels in each sample. One representative experiment of three biological replicates is shown. (C) Stage VI oocytes were co-injected with Zar1l^{Ab1} or control (Ctrl) antibodies, with mRNA encoding Flag-TRIM21 and with mRNA encoding the Flag-eGFP-cMos 3′UTR and Myc-eGFP-β-globin 3′UTR constructs. After 42 h, oocytes were treated with PG or left untreated. Oocytes from all conditions were lysed when 50% of PG-treated oocytes injected with Ctrl antibodies underwent GVBD. Samples were immunoblotted with the indicated antibodies. In parallel, RNA was isolated from the same samples and reverse transcribed, and RT-PCR against the reporter mRNAs was performed. Immunoblots and RT-PCR results of one experiment and quantification of the eGFP signal from three independent biological replicates are shown. Values were normalized to untreated oocytes injected with Ctrl antibodies and are given as mean±s.d.

we fused the ORF of Myc-eGFP to the 3′UTR of *Xenopus* β-globin, which does not undergo PG-induced cytoplasmic polyadenylation (Charlesworth et al., 2002). We injected these reporter mRNAs into stage VI oocytes that were either Ctrl or Zar1l depleted followed by PG addition (Fig. 3C). Neither Zar1l depletion nor PG treatment stimulated expression of Myc-eGFP, regulated by the β-globin 3′UTR. In contrast and as expected, PG treatment considerably increased expression of the cMos 3′UTR-controlled Flag-eGFP reporter construct in control depleted oocytes, thus validating the experimental setup. Importantly, Zar1l-depleted oocytes displayed markedly stronger expression of the cMos reporter construct already in stage VI oocytes and this was further increased upon PG treatment. Of note, in contrast to the Flag-eGFP cMos reporter, endogenous cMos did not detectably accumulate in stage VI oocytes upon Zar1l depletion. This is probably due to the reported lack of stabilizing phosphorylation of the cMos protein in stage VI oocytes (Castro et al., 2001; Matten et al., 1996; Nishizawa et al., 1992). As expected from the phenotypic analyses, prolonged expression of ectopic wild-type Zar1l also resulted in enhanced expression of the cMos reporter construct in stage VI oocytes and upon PG stimulation (Fig. S3C), further supporting the hypothesis that both the depletion and overexpression of Zar1l accelerate meiotic resumption via the same molecular pathway. Notably, the cMos reporter was expressed even more strongly upon expression of Zar1l 2Cys⁻ (Fig. S3C), which is compromised in cMos mRNA binding (Fig. 3B), and, consistently, Zar1l 2Cys⁻ accelerated meiotic resumption to a significantly greater extent than wild-type Zar1l (Fig. S3A, see Discussion). Taken together, from these experiments we concluded that Zar1l controls translational repression imposed by the cMos 3′UTR and that interfering with this function by either depleting or overexpressing Zar1l results in premature cMos expression and hence accelerated meiotic resumption.

Identification of Zar1l interaction partners by mass spectrometry

The regulated translation of mRNAs is controlled by diverse and incompletely characterized multi-protein complexes assembled on the mRNAs. To better understand how Zar1l controls translation, we determined the interactome of Zar1l by mass spectrometry (MS) analyses of Flag-Zar1l immunoprecipitated from immature oocytes. Water-injected oocytes served as controls, and both conditions, i.e. water and Flag-Zar1l mRNA-injected oocytes, were analyzed in biological triplicates. These analyses identified Zar1 and Zar2 as being associated with Zar1l, with Zar2 being considerably more enriched than Zar1 (Fig. 4). Interestingly, 4E-T/eIF4ENIF1, best known for its central function in P-bodies (Andrei et al., 2005), was among the most strongly enriched proteins. In zebrafish, 4E-T was shown to be part of the same RNP as Zar1, indicating that this interaction is conserved among the Zar family proteins and across species (Miao et al., 2017). Reportedly, 4E-T is part of a large RNP that represses translation of associated mRNAs and in somatic cells these mRNAs are stored in a deadenylated and silenced, but stable, form (Minshall et al., 2007; Rasch et al., 2020). Among the most highly enriched proteins in the Zar1l IP was eIF4E1b, which is exclusively expressed in oocytes and early embryos. Compared with the canonical eIF4E1a, eIF4E1b has reduced affinity for the 5′cap

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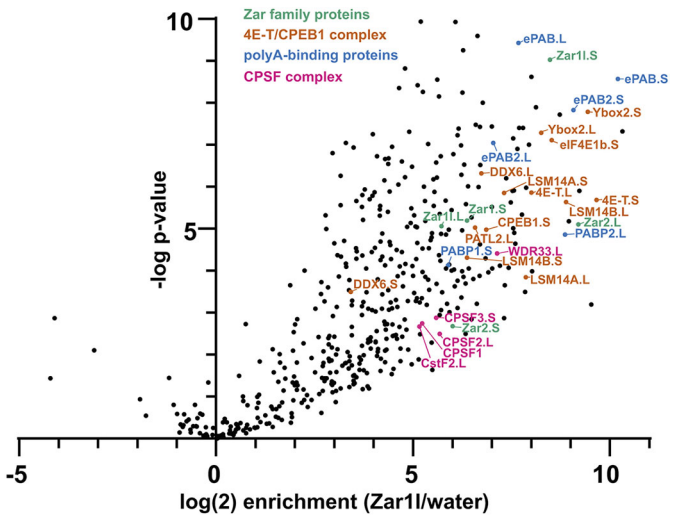

Fig. 4. Identification of Zar1l-binding partners in *Xenopus* oocytes by mass spectrometry. (A) Stage VI oocytes were injected with mRNA encoding Flag-Zar1l.S or water as control. After 18 h incubation, oocytes were lysed and subjected to anti-Flag immunoprecipitation. The lysates were not RNase-treated. Proteins were eluted from the beads and analyzed by MS. Graph shows relative enrichment [log(2) enrichment] in the Zar1l condition over the water control plotted against the corresponding *P* value [–log(10) *P* value as determined by an unpaired Student's *t*-test] of three independent biological replicates. Selected significantly enriched (s0=0.1, FDR≤0.01) proteins are indicated.

and for eIF4G, which might adjust the ability to initiate translation to the specific need of this developmental stage (Kubacka et al., 2015; Minshall et al., 2007; Villaescusa et al., 2006). Consistently, many additional proteins known to be part of the 4E-T translation-repression complex (Kamenska et al., 2016; Minshall et al., 2007; Nakamura et al., 2010; Rasch et al., 2020) interacted with Flag-Zar1l, e.g. DDX6, Ybox2, ePAB1/2, LSM14A, LSM14B, PATL2 and CPEB1. The identification of 4E-T and known 4E-T interaction partners suggests that Zar1l is a new constituent of this complex. According to the 'early oocyte repression model' (Radford et al., 2008), this 4E-T/CPEB1 translation-repressing complex is already present during early *Xenopus* oogenesis and some of its constituents, such as CPEB1 and PATL2, bind to the cMos mRNA (Cao et al., 2021; Weill et al., 2017). Notably, several subunits of the CPSF complex (CPSF1, CPSF2, CPSF3 and WDR33) (Clerici et al., 2018; Dickson et al., 1999), CstF2, which is part of a complex that assists CPSF in defining the polyadenylation site (Yang et al., 2018), and various polyA-binding proteins (e.g. PABP1, ePAB2 and ePAB) (Wilkie et al., 2005) were enriched in the Flag-Zar1l IP samples. Binding of PABPs to the polyA-tail of mRNAs protects them from nucleases and stimulates translation initiation by stabilizing the mRNA 'closed-loop' conformation (Jackson et al., 2010; Kim and Richter, 2007; Voeltz et al., 2001). Of note, ePAB has also been shown to directly bind to CPEB1 on translationally repressed mRNAs with short polyA-tails (Kim and Richter, 2007). This suggests that mRNAs bound by the Zar1l RNP could potentially be controlled by regulated polyadenylation during later developmental states. In addition to the aforementioned proteins, many of the identified Zar1l interaction partners were previously implicated in cytoplasmic granules as diverse as P-bodies, stress granules, L-bodies and the Balbiani body of early oocytes that later disperses into germ plasm islands (Boke et al., 2016; Hubstenberger et al., 2017; Markmiller et al., 2018; Neil et al., 2021), suggesting that Zar1l might be a constituent of several distinct RNPs in *Xenopus* oocytes. Of note, these MS assays were performed in the presence of endogenous mRNA, so it has to be determined by future assays whether Zar1l binds these many proteins by, direct or indirect, protein-protein interactions or whether they indirectly colocalize to the same mRNAs.

Zar1l binds to RNPs containing 4E-T and CPEB1

Given that Zar1l negatively regulates the translation of cMos mRNA, we focused our further functional analyses on the highly enriched proteins present in the 4E-T/CPEB1 complex described by the 'early oocyte repression model' (Minshall et al., 2007; Radford et al., 2008). At its core, this complex consists of 4E-T, which interacts with factors associated with the 3′UTR, and eIF4E1b bound to the 5′cap (Kamenska et al., 2016; Minshall et al., 2007; Rasch et al., 2020). To confirm the MS results, we injected stage VI oocytes with mRNA encoding Flag-Zar1l followed by anti-Flag IP. Immunoblot analyses confirmed that both the long and short isoform of 4E-T (Minshall et al., 2007) were efficiently co-precipitated with Zar1l (Fig. 5A). Consistently, CPEB1 and DDX6, additional known members of the repressive 4E-T/CPEB1 complex (Minshall et al., 2007), were also efficiently co-precipitated. These interactions were observed for both the S- and L-version of Zar1l, indicating that they have very similar cellular functions (Fig. S4A). To confirm the interaction, we performed the reciprocal IP by injecting mRNA encoding Myc-tagged 4E-T or CPEB1 into stage VI oocytes followed by anti-Myc IP. Indeed, endogenous Zar1l was co-precipitated with both proteins (Fig. 5B). Next, we analyzed the role of RNA in the interaction between Zar1l and the 4E-T/CPEB1 RNP. To this end, we expressed Flag-tagged Zar1l in stage VI oocytes and treated the lysate with RNaseA prior to IP. Interestingly, RNaseA treatment compromised co-precipitation of CPEB1 and DDX6, which suggests that their interaction with Zar1l is at least partially bridged by RNA (Fig. 5A). In contrast, the levels of associated 4E-T and ePAB were even increased, indicating that they interact with Zar1l primarily via protein-protein interactions (Fig. 5A). Endogenous Zar2 was efficiently co-precipitated with Flag-Zar1l, further validating our MS analyses. Unfortunately, owing to low sensitivity of the Zar1 antibody, we could not confirm an interaction between Flag-Zar1l and endogenous Zar1. Therefore, to investigate potential interactions among the Zar family proteins, we investigate ectopically expressed Zar1, Zar1l or Zar2 by IP from stage VI oocytes (Fig. 5C). All three proteins efficiently co-precipitated 4E-T, CPEB1, DDX6 and ePAB. This suggests that all Zar proteins can be part of the same protein complexes and this was further corroborated by the finding that endogenous Zar1l and Zar2 proteins could be co-precipitated by all ectopic Zar family proteins. None of the Zar family proteins co-precipitated the eIF4F component eIF4G, consistent with the idea that mRNAs bound by the 4E-T/CPEB1 complex are translationally repressed. Next, we immunoprecipitated endogenous Zar1l from stage VI oocytes using $Zar1l^{Ab1}$ to confirm the observed interactions for endogenous Zar1l. During these experiments, we repeatedly observed that the immunoprecipitation of Zar1l was more efficient when the lysate was treated with RNaseA before the IP (Fig. 5D). This suggests that the epitope of $Zar1l^{Ab1}$ is less accessible in the context of intact RNPs, which, in turn, implies that we could miss an interaction because a potential binding partner might bind to the same region of Zar1l as the $Zar1l^{Ab1}$ antibody. An alternative explanation could be that, in the presence of RNA, the RNPs are so large that they cannot be efficiently co-precipitated by the IP. Nevertheless, 4E-T, DDX6 and ePAB were efficiently co-precipitated with endogenous Zar1l. In line with the results obtained with ectopic Zar1l, we again

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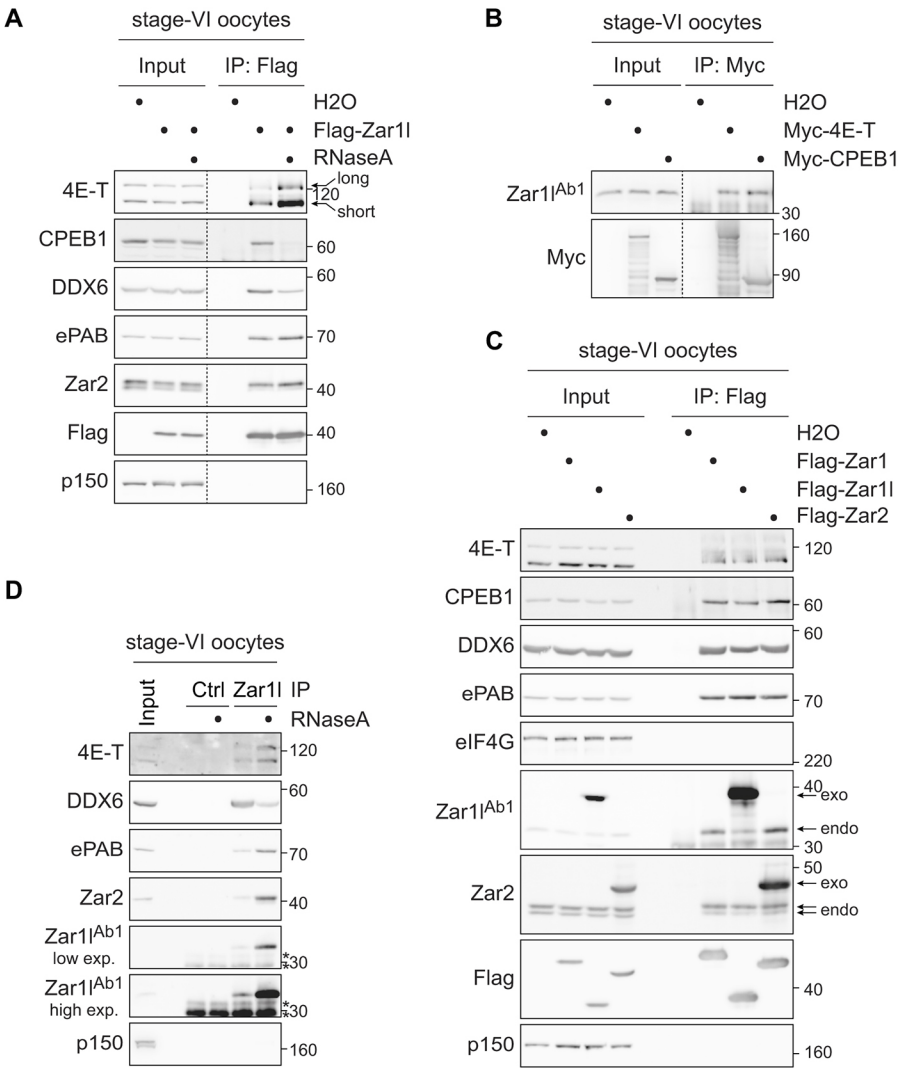

Fig. 5. Zar1l is part of a repressive RNP containing 4E-T. (A) Stage VI oocytes were injected with water or mRNA encoding Flag-Zar1l.S. After 18 h incubation, oocytes were lysed and treated with RNaseA as indicated. Oocyte lysates were subjected to anti-Flag immunoprecipitation and samples were immunoblotted as indicated. Long and short isoforms of 4E-T are indicated. Several lanes were removed at the dashed line. One representative experiment of three biological replicates is shown. (B) Stage VI oocytes were injected with water or mRNA encoding Myc-tagged 4E-T.L or CPEB1.S. After 18 h incubation, oocyte lysates were subjected to α-Myc immunoprecipitation and samples were immunoblotted with the indicated antibodies. Several lanes were removed at the dashed line. One representative experiment of three biological replicates is shown. (C) Stage VI oocytes were injected with water or mRNA encoding Flag-tagged Zar1.S, Zar1l.S or Zar2.S. After 18 h incubation, oocyte lysates were subjected to anti-Flag immunoprecipitation and samples were immunoblotted with the indicated antibodies. Arrows indicate endogenous and exogenous Zar1l and Zar2 proteins. One representative experiment of three biological replicates is shown. (D) Stage VI oocytes were lysed and treated with RNaseA as indicated. Oocyte lysates were subjected to immunoprecipitation with Zar1l^{Ab1} or unspecific control (Ctrl) antibodies and samples were immunoblotted with the indicated antibodies. Low and high exposure immunoblots are shown for Zar1l. Asterisks indicate unspecific bands. One representative experiment of three biological replicates is shown.

observed that the interaction with 4E-T and ePAB was not sensitive to RNaseA treatment, whereas the interaction with DDX6 was significantly weakened by it. Notably, we also co-precipitated endogenous Zar2 with endogenous Zar1l, thus confirming that they localize to the same RNPs in stage VI oocytes. Taken together, our results suggest that Zar1l is part of large RNPs that contain the translation repressor 4E-T and the critical polyadenylation regulator CPEB1.

Zar1l controls 4E-T association to CPEB1

Finally, we aimed to further understand how Zar1l controls translation of cMos mRNA. Reportedly, timing of cMos expression during meiotic maturation is primarily controlled by CPEB1, which binds to mRNAs containing a CPE in their 3′UTR and serves as a hub that, dependent on the developmental stage, recruits translation-repressing and -activating components (Radford et al., 2008; Weill et al., 2017). As Zar1l depletion accelerates cMos expression, we investigated the function of Zar1l in the recruitment of the translation repressor 4E-T to the CPEB1 complex. PG-induced meiotic resumption is accompanied by an early partial degradation of Zar1l (Fig. 1D,E). To test whether this initial limited degradation of Zar1l affects the recruitment of 4E-T to CPEB1, we immunoprecipitated ectopic CPEB1 from oocytes at distinct time points after PG stimulation. As seen before, Zar1l was partially degraded within a time window after PG treatment where Cdk1 is still inactive (Fig. 6A, see 2 h time point). As expected, decreased Zar1l levels were mirrored by reduced binding of Zar1l to CPEB1. Importantly, this coincided with lower association of 4E-T with CPEB1, suggesting that partial degradation of Zar1l is associated with considerable remodeling of the CPEB1 RNP well before the oocytes undergo GVBD. If this applies, experimental reduction of Zar1l levels should also result in reduced binding of 4E-T to CPEB1. To test this, we again used TRIM-Away to deplete Zar1l in stage VI oocytes and analyzed the amount of 4E-T co-precipitating with Myc-tagged CPEB1. Compared with Ctrl-depleted oocytes, significantly less 4E-T associated with Myc-CPEB1 upon Zar1l depletion (Fig. 6B). Of note, Zar1l depletion was associated with reduced levels of 4E-T (see input). Therefore, we cannot unambiguously distinguish cause and consequence, i.e. are the reduced total levels of 4E-T the cause for reduced binding of 4E-T to CPEB1 or is the reduced binding of 4E-T to CPEB1 the cause for its destabilization. Irrespective of the underlying mechanism, these data support our hypothesis that the translation repressor 4E-T is released from CPEB1 when Zar1l levels are reduced, resulting in premature expression of cMos and hence accelerated meiotic resumption. Next, we tested whether the overexpression of Zar1l, which phenocopied the Zar1l depletion in that it induced premature cMos expression and meiotic maturation (see Fig. S3A,C), also

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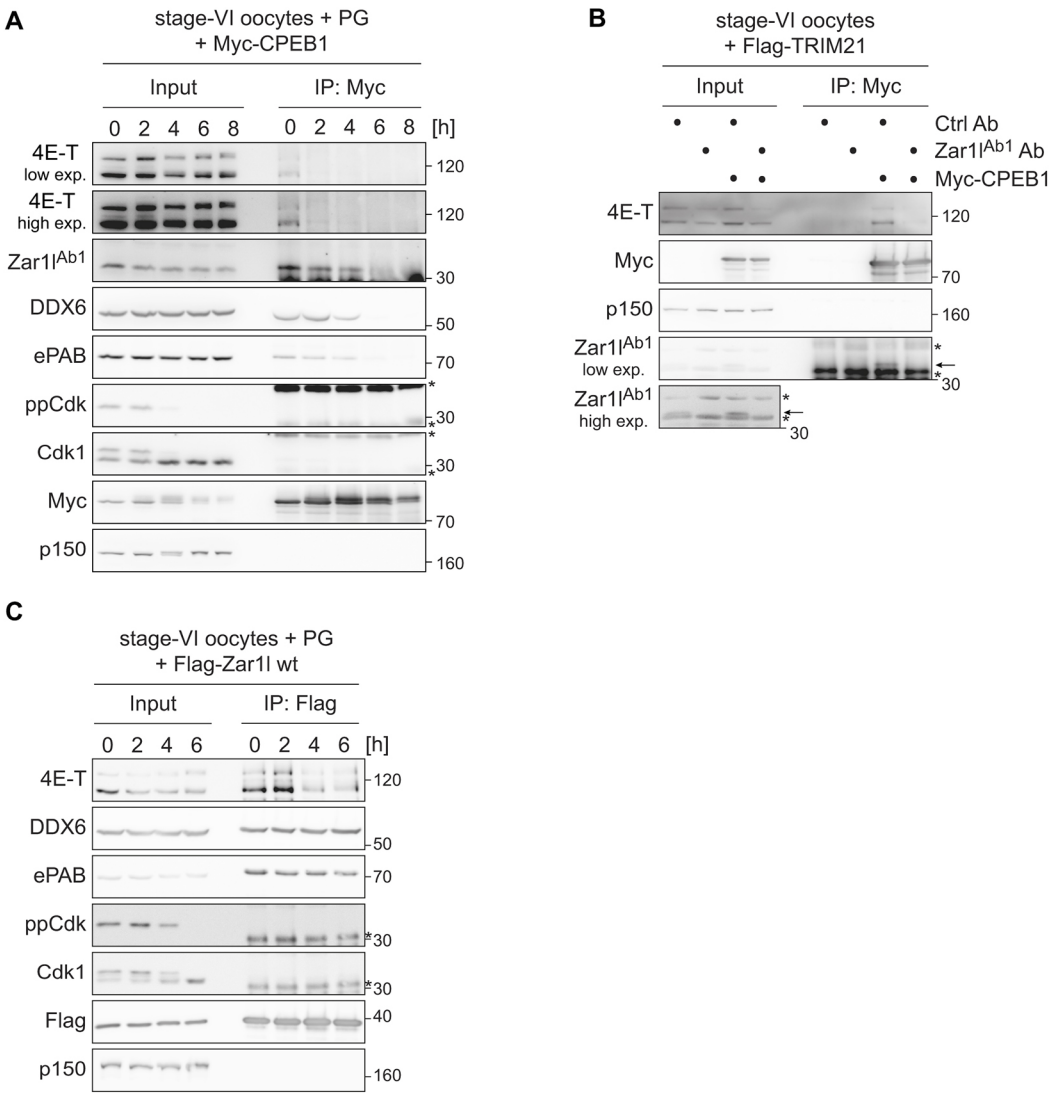

Fig. 6. Zar1l affects the association between CPEB1 and 4E-T. (A) Stage VI oocytes were injected with mRNA encoding Myc-CPEB1.S. After 18 h, oocytes were treated with PG and lysed at the indicated time points. Oocyte lysates were subjected to anti-Myc immunoprecipitation and samples were immunoblotted as indicated. Low and high exposure immunoblots are shown for 4E-T. Asterisks indicate unspecific bands. One representative experiment of three biological replicates is shown. (B) Stage VI oocytes were co-injected with Zar1l^{Ab1} or unspecific control (Ctrl) antibodies, mRNA encoding Flag-TRIM21 and mRNA encoding Myc-CPEB1.S. After 42 h, oocyte lysates were subjected to anti-Myc immunoprecipitation and samples were immunoblotted as indicated. Low and high exposure immunoblots are shown for Zar1l. Arrows indicate Zar1l. Asterisks indicate unspecific bands. One representative experiment of three biological replicates is shown. (C) Stage VI oocytes were injected with mRNA encoding Flag-Zar1l.S. After 18 h incubation, oocytes were treated with PG and lysed at the indicated time points. Oocyte lysates were subjected to anti-Flag immunoprecipitation and samples were immunoblotted with the indicated antibodies. Asterisks indicate unspecific bands. One representative experiment of three biological replicates is shown.

destabilizes the 4E-T/CPEB1 complex. To this end, we immunoprecipitated 4E-T from stage VI oocytes after prolonged overexpression of Zar1l wild type or 2Cys^{-} (Fig. S5A). We observed that the overexpression of both Zar1l variants resulted in a destabilization of CPEB1, which consequentially led to a decrease in the amount of 4E-T-associated CPEB1. Thus, although both the depletion and overexpression of Zar1l result in premature cMos expression by deregulating the 4E-T/CPEB1 complex, the molecular mechanism seems to differ in that they predominantly destabilize 4E-T and CPEB1, respectively. Finally, we investigated whether 4E-T binding to Zar1l is also controlled by a mechanism that is temporally uncoupled from Zar1l degradation and could be employed to regulate RNPs bound by the persisting pool of Zar1l during later stages of meiotic maturation. Ectopic expression of Flag-tagged Zar1l in *Xenopus* oocytes was sufficient to override the degradation normally observed for endogenous Zar1l upon PG stimulation (Fig. 6C). Interestingly, despite equal levels of Flag-Zar1l being immunoprecipitated at distinct time points after PG treatment, the levels of co-precipitated 4E-T sharply dropped around 4 h post PG-treatment, a time point when Cdk1 becomes active (see ppCdk1 blot). This suggests that the partial degradation of Zar1l early after the PG stimulus is the main driver for remodeling its associated 4E-T/CPEB1 RNP and that other processes, such as post-translational modifications and/or the degradation of other constituents, modulate the remaining fraction of Zar1l RNPs during later stages of meiotic maturation.

DISCUSSION

Here, we have elucidated the function of Zar1l, a yet uncharacterized member of the Zar protein family, during female

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meiosis of *Xenopus laevis*. We show by MS and immunoblot assays that Zar1l is a constituent of the previously described 4E-T/CPEB1 complex (Minshall et al., 2007). mRNAs bound to 4E-T RNPs were described to be in a silent but stable state (Rasch et al., 2020). Our MS data support this model because the Zar1l IP was not enriched for proteins associated with actively translated 'closed loop' mRNAs (e.g. eIF4G) or for decapping factors (e.g. Dcp1 and Dcp2) (Christou-Kent et al., 2020). We further show that Zar1l directly binds to the cMos mRNA and its depletion induces precocious cMos expression upon hormone stimulation and, consequentially, accelerated meiotic resumption. Thus, in *Xenopus* oocytes, Zar1l, like Zar1 and Zar2 (Yamamoto et al., 2013), seems to have a translation-repressing function. Previous studies in mice have revealed that knockout of Zar1 and Zar2 resulted in drastic meiotic defects and infertility (Rong et al., 2019). Notably, mouse Zar1/Zar2 double knockout oocytes showed delayed meiotic maturation, which is contrary to the acceleration we observed in Zar1l-depleted *Xenopus* oocytes. One explanation could be that *Xenopus* and mouse Zar proteins are functionally distinct in that the former repress translation, while the latter promote it. Indeed, polyadenylation and translation of mRNAs encoding crucial meiotic regulators, such as cyclin B1, Btg4 or Tpx2, were inefficient in Zar1/Zar2 knockout mouse oocytes, pointing towards a translation-activating function (Rong et al., 2019). Although we cannot exclude the possibility that *Xenopus* and mouse Zar proteins have opposing effects on translation, one has to consider the timing of Zar depletion. We depleted Zar1l from prophase-arrested stage VI oocytes and therefore did not interfere with proteins translated during early oogenesis. In contrast, Zar1/Zar2 double knockout mice developed oocytes in the complete absence of Zar1/Zar2 and, accordingly, the accumulation of hundreds of mRNAs was affected during early oogenesis in these oocytes (Rong et al., 2019). Such early effects could interfere with late functions of Zar1 or Zar2; therefore, solid conclusions on the function of Zar proteins in different species require the depletion of the proteins at identical developmental stages. Irrespective of the function of mouse Zar proteins, we observed that Zar1l depletion increased cMos translation in *Xenopus* oocytes upon PG stimulation. However, this increase in cMos expression, involving the 3′UTR of cMos as shown by our reporter construct assay (Fig. 3C), was not sufficient for spontaneous PG-independent meiotic resumption, indicating that hormone treatment induces further stimulatory pathways. Potential stimulatory mechanisms could be the phosphorylation-dependent stabilization of the cMos protein (Castro et al., 2001; Matten et al., 1996; Nishizawa et al., 1992) or the phosphorylation of CPEB1 by RINGO-Cdk1 and Aurora A (Kim and Richter, 2007; Mendez et al., 2000). Immunoprecipitation of wild-type Zar1l followed by semi-quantitative RT-PCR suggests that Zar1l binds to the cMos mRNA (Fig. 3A,B). Binding of Zar1l to cMos mRNA seems to be direct, as mutations of conserved cysteine residues (2Cys^{-}) in the putative RNA-binding domain significantly reduced the amount of associated cMos mRNA (Fig. 3B). Notably, Zar1l 2Cys^{-} upon prolonged expression further aggravated the effect on enhanced expression of cMos and the Flag-eGFP-cMos 3′UTR reporter construct compared with wild-type Zar1l (Fig. S3A,C); consequentially, oocytes expressing Zar1l 2Cys^{-} resumed meiosis significantly faster than wild-type Zar1l-expressing oocytes (Fig. S3A). We speculate that although overexpression of both Zar1l wild type and 2Cys^{-} deregulates the 4E-T/CPEB1 complex (Fig. S5A), Zar1l 2Cys^{-} (which is compromised in mRNA binding, but apparently competent in protein-protein interactions) additionally acts in a dominant-negative manner by titrating inhibitory RNA-binding proteins away from mRNA-encoding meiosis promoting regulators, e.g. cMos. Given that Zar1 and Zar2 bind to TCS sequences present in the 3′UTR of mRNAs (Yamamoto et al., 2013) and the very high degree of sequence conservation in the C-terminal RNA-binding domain of the Zar family proteins, it is tempting to speculate that Zar1l also binds to TCS elements. If this applies, further studies will be required to analyze whether and how the different Zar proteins select the TCS sequences in their target RNAs, especially considering that all Zar proteins share a very similar network of interactors and are probably localized to the same subset of RNPs (Fig. 5). Reduction of Zar1l levels, either physiologically by PG stimulation or artificially by TRIM-Away depletion, resulted in reduced binding of 4E-T to CPEB1 (Fig. 6A,B). Under physiological conditions, Zar1l destabilization and a decreased interaction between 4E-T and CPEB1 occurred very shortly after PG stimulation at a time where Cdk1 is not yet active (Fig. 6A). Yet if we compensated for PG-induced Zar1l degradation by

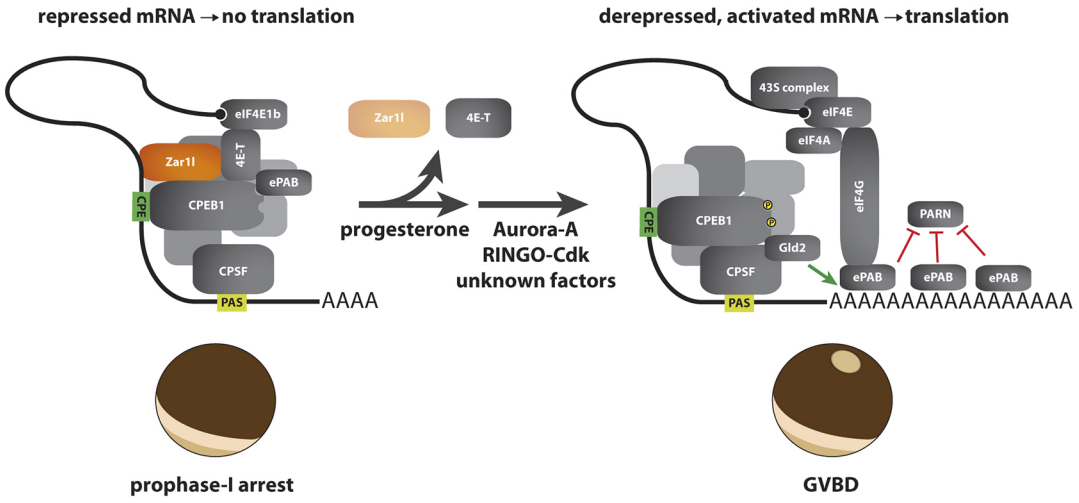

Fig. 7. Working model for the function of Zar1l during meiosis in *Xenopus laevis*. Control of the mRNA translation status by Zar1l during prophase I arrest (left) and after meiotic resumption (right) in oocytes of *Xenopus laevis*.

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expressing ectopic Zar1l, the ability of 4E-T to bind Zar1l declined around the time of Cdk1 activation (Fig. 6C). Based on these data, we propose the following working model. During the prolonged prophase I arrest, mRNAs bound to the 4E-T/CPEB1/Zar1l RNP are kept in a silent and deadenylated, yet stable, state (Fig. 7). Hormonal stimulation triggers a rapid but limited degradation of Zar1l, which partially liberates 4E-T from the CPEB1 RNP (Fig. 6A). This probably results in considerable remodeling of the repressed RNP, with some components being released and others remaining associated and participating in active translation of the associated mRNA, which has, for example, been shown for the *C. elegans* eIF4E isoform IFE-3 in transiently repressed RNPs containing the 4E-T orthologue IFET-1 (Huggins et al., 2020; Sengupta et al., 2013). Although dissociation of 4E-T is associated with the loss of the tight inhibitory state of the RNP, further positive stimuli are required to fully activate polyadenylation and thus translation of specific mRNAs needed for induction of meiotic maturation. The precise polyadenylation timing of individual mRNAs is then probably further adjusted by the combinatorial code of cis-acting mRNA elements in their 3′UTRs bound to trans-acting protein factors such as Musashi and Pumilio (Christou-Kent et al., 2020). After Cdk1 activation and GVBD, 4E-T might then be further removed from the persisting pool of Zar1l (Fig. 6C). Further RNA-seq studies will be essential to obtain a comprehensive picture of mRNAs whose translational activation at distinct time points of meiotic maturation is controlled by Zar1l and the other members of the Zar protein family.

Orthologs of 4E-T and remodeling of their RNPs to control the translational state of associated mRNAs have been studied in other species. Analogous to *Xenopus* 4E-T, these orthologues bind eIF4E to displace eIF4G and require at least one other protein for efficient mRNA binding, e.g. the *Drosophila* 4E-T protein Cup binds to mRNA 3′UTRs through the RNA-binding proteins Bruno or Smaug (Huggins and Keiper, 2020; Nakamura et al., 2004; Nelson et al., 2004). As seen for the Zar1l-bound 4E-T/CPEB1 RNP here, these RNPs can be dynamically remodeled to activate mRNA translation at the correct time and/or place in the cell, e.g. Cup is dissociated from the Oskar mRNA at the posterior pole of the oocyte (Kugler and Lasko, 2009). Overall, this suggests that in *Xenopus* oocytes 4E-T RNPs containing Zar1l follow a developmentally conserved general mechanism of dynamic mRNA repression that is modified and used in a species specific manner (Huggins and Keiper, 2020).

MATERIALS AND METHODS

Preparation of stage VI oocytes

Xenopus laevis frogs were bred and maintained at the animal research facility, University of Konstanz, according to the regulations by the Regional Commission, Freiburg, Germany (Az. 35-9185.81/G-17/121). Ovaries surgically removed from mature frogs were incubated in 1×MBS [5 mM HEPES (pH 7.8), 88 mM NaCl, 1 mM KCl, 1 mM $MgSO_4$, 2.5 mM $NaHCO_3$ and 0.7 mM $CaCl_2$] supplemented with 50 ng/µl Liberase (Roche) at 23°C for 90 min. After extensive washing in 1×MBS, stage VI oocytes were collected and kept at 19°C until further treatment.

Oocyte lysis and time course experiments

Stage VI oocytes were treated with 5 ng/µl PG (Sigma-Aldrich) in 1×OR2 [5 mM HEPES (pH 7.8), 82.5 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 1 mM Na_2HPO_4] and incubated at 23°C. When indicated, the buffer was additionally supplemented with 50 µM U0126 (Promega). To analyze meiotic timing, oocytes were imaged under a Stemi 2000-C (Zeiss) with a SPOT Insight 2MP Color camera. For immunoblotting, oocytes were lysed by mechanical shearing in 5 µl lysis buffer per oocyte [137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 5 mM β-glycerophosphate, 2 mM NaF and 1× cOmplete Protease Inhibitor Cocktail (Sigma-Aldrich) (pH=7.4)]. The lysate was centrifuged at 20,000 ***g*** for 10 min at 4°C and the clear supernatant was transferred to one volume of 3×Laemmli sample buffer [LSB; 180 mM Tris (pH 6.8), 10% SDS, 30% glycerol, 15% β-mercaptoethanol].

Microinjection of stage VI oocytes

Needles for microinjection were pulled from glass capillaries (World Precision Instruments, 504949) with a P-97 Micropipette Puller (Sutter Instrument) and cut manually to the desired length. Typically, volumes between 9.2 and 18.4 nl were injected into stage VI oocytes using a Nanoliter 2010 Microinjection Pump (World Precision Instruments).

IVT and mRNA production

Coupled *in vitro* transcription and translation was performed with the TNT SP6 High-Yield Wheat Germ Protein Expression System (Promega). mRNA was produced with the mMESSAGE mMACHINE T7 Ultra Transcription Kit (Thermo Fisher). All mRNAs were polyadenylated, except for the Flag-eGFP_cMos 3′UTR and Myc-eGFP_β-globin 3′UTR mRNAs.

DNA constructs

DNA primers used for cloning can be found in Table S1. The following constructs were used in this study: Flag-Zar1.L (*Xenopus laevis*, N-terminal 3xFlag-tag, wild type); Flag-Zar1.S (*Xenopus laevis*, N-terminal 3xFlag-tag, wild type); Flag-Zar1l.L (*Xenopus laevis*, N-terminal 3xFlag-tag, wild type); Flag-Zar1l.S (*Xenopus laevis*, N-terminal 3xFlag-tag, wild type); Flag-Zar1l.S 2Cys^{-} (*Xenopus laevis*, N-terminal 3xFlag-tag, Cys190Ala Cys217Ala); Flag-Zar2.L (*Xenopus laevis*, N-terminal 3xFlag-tag, wild type); Flag-Zar2.S (*Xenopus laevis*, N-terminal 3xFlag-tag, wild type); Myc-4E-T.L (*Xenopus laevis*, N-terminal 6xMyc-tag, wild type); Myc-CPEB1.S (*Xenopus laevis*, N-terminal 6xMyc-tag, wild type); Flag-TRIM21 (*Mus musculus*, codon-optimized for *Xenopus laevis*, N-terminal 3xFlag, amino acid Ser2-Met462, synthesized by Thermo Fisher); Flag-TRIM21$^{\Delta C}$ (*Mus musculus*, codon-optimized for *Xenopus laevis*, N-terminal 3xFlag, amino acid Ser2-Cys281); Flag-eGFP_cMos 3′UTR (*Xenopus laevis*, 3xFlag-eGFP followed by complete 3′UTR of cMos.L with five adenylyl residues added at the 3′end); and Myc-eGFP_β-globin 3′UTR (*Xenopus laevis*, 6xMyc-eGFP followed by complete 3′UTR of Hbg1.L with five adenylyl residues added at the 3′end)

Antibodies

Zar1l^{Ab1} antibody (1:2000) was generated by immunizing rabbits with a mixture of peptides KPKQPYWKPPYKC and KPKQQYWKPPYKC and purification against the same peptides. Zar1l^{Ab2} antibody (1:1000) was generated by immunizing rabbits with the peptide CLGPEFGLGRRFT-KEVG and purification against the same peptide. Zar1 antibody (1:500) was generated by immunizing rabbits with the peptide KGMSWRQKNYLA-SYGDTGDYC and purification against the same peptide. Zar2 antibody (1:2000) was generated by immunizing rabbits with the peptide FPKNKQAAWKSNKSSEC and purification against the same peptide. ePAB antibody (1:1000) was generated by immunizing rabbits with the peptide DGIDDDRLRKEFC and purification against the same peptide (characterization in Fig. S6A). 4E-T^{Ab2} was generated by immunizing rabbits with the peptide DRDVRGGEKDREPREGRDREKEYKDKRC and purification against the same peptide. The following antibodies were purchased from commercial suppliers: Flag-tag antibody (Sigma-Aldrich F1804, 1:1000); CPEB1 antibody (Biozol MBS9213514, 1:500); cMos antibody (Santa Cruz sc-86, 1:500); ppMAPK antibody (Cell Signaling 9106, 1:2000); MAPK antibody (Santa Cruz sc-154, 1:2000); Cdk1 antibody (Santa Cruz sc-54, 1:500); p150glued antibody (BD Transduction Laboratories 610473, 1:1000); 4E-T antibody (Cell Signaling 2297, 1:500); DDX6 antibody (Novus Biologicals NB200-191, 1:1000); eIF4G antibody (Cell Signaling 2469, 1:2000); GFP antibody (Thermo Fisher MA5-15256, 1:1000); rabbit Ctrl antibody (Biozol GSC-A01008). Myc antibody (1:100) was purified from hybridoma cells (9E10). ppCdk (phospho-Thr14

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phospho-Tyr15) antibody (1:500) was a gift from Tim Hunt (Cancer Research UK, Clare Hall Laboratories, UK). Cdc27 (1:1000) and XErp1 (1:500) antibodies have been described previously (Tischer et al., 2012). HRP-coupled anti-rabbit (711-005-152, 1:10,000) and anti-mouse (115-035-146, 1:10,000) secondary antibodies were purchased from Dianova. In Fig. 5D, all primary antibodies derived from rabbit were detected using a conformation-specific secondary antibody purchased from Cell Signaling (5127, 1:5000).

TRIM-Away of Zar1l in *Xenopus* oocytes

Stage VI oocytes were injected with 4.6 ng mRNA encoding Flag-TRIM21 or Flag-TRIM21$^{\Delta C}$ and 11.5 ng control or α-Zar1l antibodies. Unless otherwise stated, oocytes were incubated in 1×MBS at 19°C for 42 h.

Zar1l overexpression

Stage VI oocytes were injected with water or 5.5 ng mRNA encoding Flag-Zar1l.S wild type or 2Cys$^-$ and incubated in 1×MBS for 42 h at 19°C. Oocytes were subjected to 4E-T immunoprecipitation as described below or treated with 5 ng/µl PG in 1×OR2 and incubated at 23°C. Oocytes were lysed at the indicated time points and meiotic timing was analyzed as described above.

Immunoprecipitation

For immunoprecipitation of endogenous Zar1l, oocyte lysates were prepared as described above. As indicated, 100 ng/µl RNaseA (Roth) were added. All other conditions were treated with 100 U/ml RNasin Ribonuclease Inhibitor (Promega). 150 µl oocyte lysate were added to 10 µg α-Zar1l^{Ab1} antibody coupled to Dynabeads Protein G (Thermo Fisher Scientific) and incubated for 1.5 h at 6°C. Beads were washed three times with 1× wash buffer (WB) [20 mM Tris, 300 mM NaCl, 0.2% Tween20, 1 mM EDTA, 1 mM EGTA (pH 8.0)] and resuspended in 30 µl 1.5×LSB.

For immunoprecipitation of ectopic Flag-Zar1.S, Flag-Zar1l.S, Flag-Zar1l.L and Flag-Zar2.S, oocytes were injected with 5.5 ng mRNA and incubated in 1×MBS for 18 h at 19°C. Samples were treated with 5 ng/µl PG (Sigma-Aldrich) as indicated and incubated at 23°C in 1×OR2. Oocytes were lysed as described above. As indicated, 100 ng/µl RNaseA (Roth) was added. All other conditions were treated with 100 U/ml RNasin Ribonuclease Inhibitor (Promega). 100 µl oocyte lysate were added to 4 µg α-Flag antibody coupled to Dynabeads Protein G (Thermo Fisher Scientific) and incubated for 1.5 h at 6°C. Beads were washed three times with 1×WB buffer and resuspended in 30 µl 1.5×LSB.

For immunoprecipitation of ectopic Myc-CPEB1.S and Myc-4E-T.L, oocytes were injected with 11 ng mRNA and incubated in 1×MBS for 18 h at 19°C. For experiments including simultaneous Zar1l TRIM-Away, 5.5 ng Myc-CPEB1.S mRNA were injected and oocytes were incubated in 1×MBS for 42 h at 19°C. Samples were treated with 5 ng/µl PG (Sigma-Aldrich) as indicated and incubated at 23°C in 1×OR2. Oocytes were lysed in lysis buffer supplemented with 100 U/ml RNasin Ribonuclease Inhibitor (Promega). 150 µl oocyte lysate were added to 12 µg anti-Myc antibody coupled to Dynabeads Protein G (Thermo Fisher Scientific) and incubated for 1.5 h at 6°C. Beads were washed three times with 1×WB buffer and resuspended in 30 µl 1.5×LSB.

For immunoprecipitation of endogenous 4E-T, antibodies were covalently crosslinked to Dynabeads Protein G (Thermo Fisher Scientific) with DMP (Sigma-Aldrich) as described for the mass spectrometry experiment in the supplementary Materials and Methods. Zar1l wild type or 2Cys$^-$ were overexpressed in oocytes as described above. Oocytes were lysed in Lysis Buffer supplemented with 100 U/ml RNasin Ribonuclease Inhibitor (Promega). 150 µl oocyte lysate were added to 5 µg α-4E-T^{Ab2} or unspecific Ctrl antibody as indicated and incubated for 1.5 h at 6°C. Beads were washed three times with 1xWB Buffer and resuspended in 30 µl 1.5x LSB.

Mass spectrometry

A detailed description of the mass spectrometry experiment and analysis (Fig. 4) can be found in the supplementary Materials and Methods.

RNA immunoprecipitation

Stage VI oocytes were injected with water or 5.5 ng mRNA encoding Flag-Zar1l.S wild type and 2Cys$^-$ and incubated in 1×MBS for 18 h at 19°C. Pools of 50 oocytes were lysed in 250 µl lysis buffer supplemented with 100 U/ml RNasin Ribonuclease Inhibitor (Promega). 5 µl lysate were added to 5 µl 3×LSB as input for immunoblotting while 10 µl lysate were added to 150 µl QIAzol (Qiagen) as input for RNA isolation. The residual lysate was added to 10 µg anti-Flag antibody coupled to Dynabeads Protein G (Thermo Fisher Scientific) and incubated for 1.5 h at 6°C. Beads were washed three times with 1×WB Buffer. 1/30 of the Dynabeads were used for immunoblotting. The residual Dynabeads were resuspended in 150 µl QIAzol (QIAGEN). Total RNA was isolated from input and Dynabead samples with RNeasy Mini Kit (Qiagen). cDNA was synthesized with Transcriptor High Fidelity cDNA Synthesis Kit (Roche) using Random Hexamer primers. PCR was performed using the PfuUltra II Fusion HS DNA Polymerase (Agilent) with the following parameters: 3 min at 96°C then 30 cycles of 1 min at 96°C, 30 s at 60°C, 30 s at 72°C, then 8 min at 72°C. Primer pairs specific for cMos.L 3′UTR (pair 1, ATCACAGGCTTCCACTCCAC and CACAGGAAAAAGCGACCAAT; pair 2, TGCAAACATCTCTGGCAGTC and CCCAGGGACATTGCTG-TATT) were designed by Primer3Plus (Untergasser et al., 2007).

cMos 3′UTR reporter assay

The complete cMos.L 3′UTR followed by five adenylyl residues was amplified from cDNA of stage VI oocytes using the primers ATTATCTAGACGTCCAGAACAGGGAGC and TAATTCTAGATTTT-TAGACAAATCAATTTCTTTATTATAAAACTATATATTCACATATG. The complete β-globin (Hbg1.L) 3′UTR followed by five adenylyl residues was amplified from cDNA of stage VI oocytes using the primers ATTATCTAGAACCAGCCTCAAGAACACCC and TAATTCTAGATT-TTTGTGAAGAAACTTTCTTTTTATTAGGAGCAG. The PCR products were cloned downstream of the 3xFlag-eGFP or the 6xMyc-eGFP ORF, respectively, in a pCS2 vector. The templates for the *in vitro* transcription of 3xFlag-eGFP_cMos 3′UTR and 6xMyc-eGFP_β-globin 3′UTR were amplified from these plasmids by PCR. PCR primers were chosen to add either 30 adenylyl residues (Fig. 3C) or five adenylyl residues (Fig. S3C) at the end of the 3′UTR. Stage VI oocytes were injected with 0.46 ng of the 3xFlag-eGFP_cMos 3′UTR mRNA. In Fig. 3C, oocytes were co-injected with 0.46 ng 6xMyc-eGFP_β-globin 3′UTR mRNA. 4.6 ng Flag-TRIM21 mRNA and 11.5 ng antibodies (Fig. 3C) or 5.5 ng Flag-Zar1l.S wild type/2Cys$^-$ mRNA (in Fig. S3C) were co-injected as indicated. The oocytes were incubated in 1×MBS for 42 h at 19°C. Subsequently, the oocytes were transferred to 1×OR2, treated with 5 ng/µl PG as indicated and incubated at 23°C. For RT-PCR, 10 µl oocyte lysate were resuspended in 150 µl QIAzol (Qiagen). Total RNA was isolated with RNeasy Mini Kit (Qiagen). cDNA was synthesized with Transcriptor High Fidelity cDNA Synthesis Kit (Roche) using Random Hexamer primers. PCR was performed using the PfuUltra II Fusion HS DNA Polymerase (Agilent) with the following parameters: 3 min at 96°C then 30 cycles of 1 min at 96°C, 30 s at 60°C and 30 s at 72°C then 8 min at 72°C. Primer pairs specific for the 3xFlag-eGFP_Mos 3′UTR reporter (TGTTCTTTTTGCAG-GATCCAC and GGCCTTTGTCGTCATCATCT) and the 6xMyc-eGFP_β-globin 3′UTR reporter (TCTTTTTGCAGGATCCCATC and CC-CAAGCTCTCCATTTCATT) mRNAs were designed with Primer3Plus (Untergasser et al., 2007).

Statistical analysis

Amino acid identity between *Xenopus laevis* Zar proteins was calculated by aligning sequences [Xl_Zar1.L (XB-GENE-17331915), Xl_Zar1.S (XB-GENE-942430), Xl_Zar1l.L (XP_018103300.1), Xl_Zar1l.S (XP_018105973.1), Xl_Zar2.L (XP_018103181.1) and Xl_Zar2.S (NP_001153159.1)] on uniprot.org. Sequence alignments were generated using Jalview v2.11.2.3 (Waterhouse et al., 2009). Heat maps were generated using GraphPad Prism 9. Band intensities in immunoblots were quantified using ImageJ and values are given as mean±s.d. No statistical method was used to predetermine sample size. Oocytes were randomly assigned to experimental groups. Investigators were not blinded during data

collection and analysis. No experiments or data points were excluded from the analyses.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: A.H., T.U.M.; Methodology: A.H.; Formal analysis: A.H., M.L.N.; Investigation: A.H., M.L.N.; Data curation: M.L.N.; Writing - original draft: A.H., T.U.M.; Writing - review & editing: A.H., M.L.N., F.S., T.U.M.; Visualization: A.H.; Supervision: F.S., T.U.M.; Funding acquisition: F.S., T.U.M.

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Data availability
The mass spectrometry proteomics data have been deposited in the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2022) partner repository with the dataset identifier PXD032170.

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