Supplementary Information

Non-canonical translation via deadenylated 3'UTRs maintains primordial germ cells

Youngnam N. Jin[1,2,3,4,6,7,9,*], Peter J. Schlueter[1,2,3,9], Nathalie Jurisch-Yaksi[1,2,3], Pui-Ying Lam[1,2,3,4,6,7], Shan Jin[1,2,3], Woong Y. Hwang[1,2], Jing-Ruey Joanna Yeh[1,2,3], Masaaki Yoshigi[5], Shao-En Ong[6,8], Monica Schenone[6], Christina R. Hartigan[6], Steven A. Carr[6], & Randall T. Peterson[1,2,3,4,6,7,*]

[1]Cardiovascular Research Center, Massachusetts General Hospital, Charlestown, Massachusetts, USA.

[2]Department of Medicine, Massachusetts General Hospital, Charlestown, Massachusetts, USA.

[3]Department of Medicine, Harvard Medical School, Boston, Massachusetts, USA.

[4]Department of Systems Biology, Harvard Medical School, Boston, Massachusetts, USA.

[5]Departments of Pediatrics, University of Utah, Salt Lake City, Utah, USA.

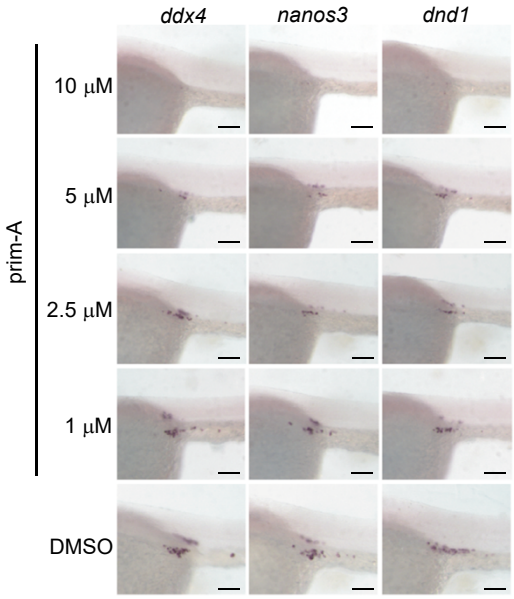
[6]Broad Institute, Cambridge, Massachusetts, USA.

[7]Present address: College of Pharmacy, University of Utah, Salt Lake City, Utah, USA.

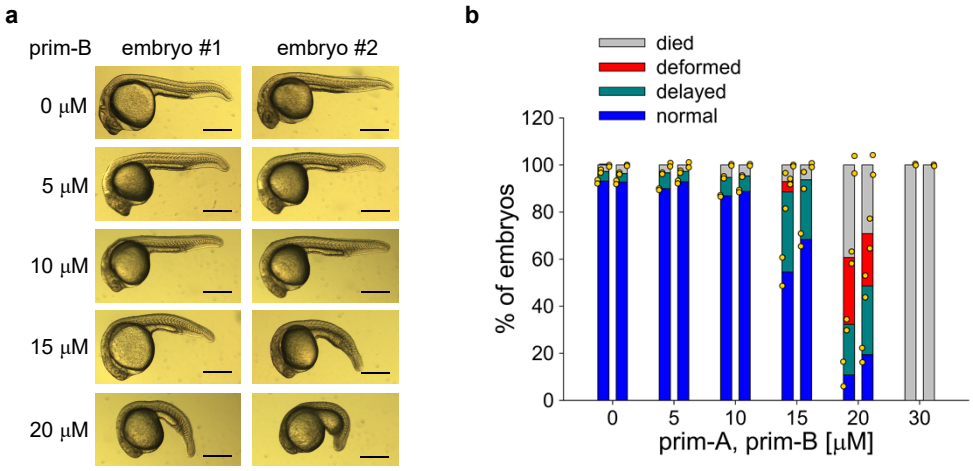
[8]Present address: Department of Pharmacology, University of Washington, Seattle, Washington, USA.

[9]These authors contributed equally to this work.

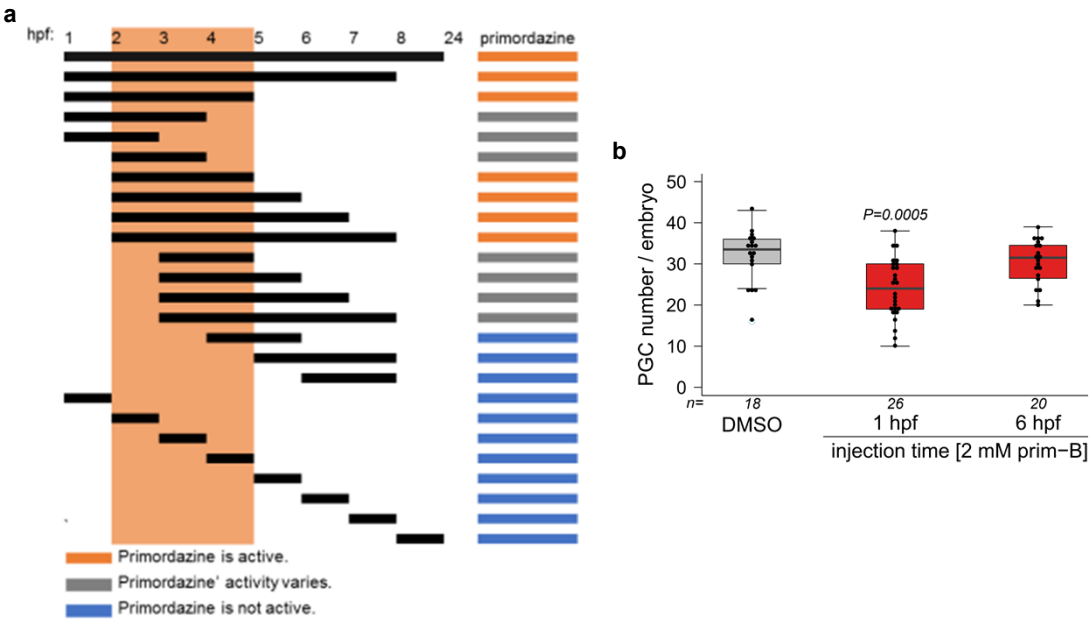
*e-mail: youngnam.jin@pharm.utah.edu or randall.peterson@pharm.utah.edu



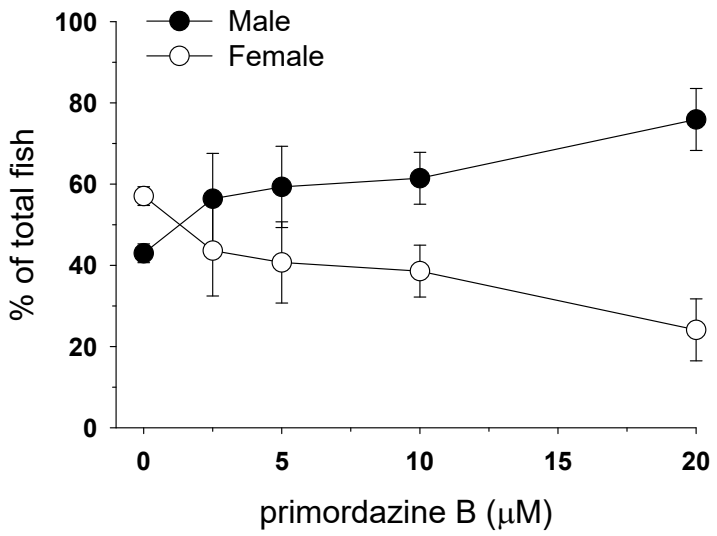
Supplementary Figure 1: Primordazine reduces PGC numbers in a dose dependent manner. Primordazine reduces PGC numbers in a dose dependent manner. WISH with three PGC markers, *ddx4*, *nanos3*, and *dnd1*, shows decreased numbers of PGCs by prim-A treatment at as low as 2.5 μM and a dramatic loss at 10 μM. This experiment was repeated two times with similar results. Scale bar = 100 μm.



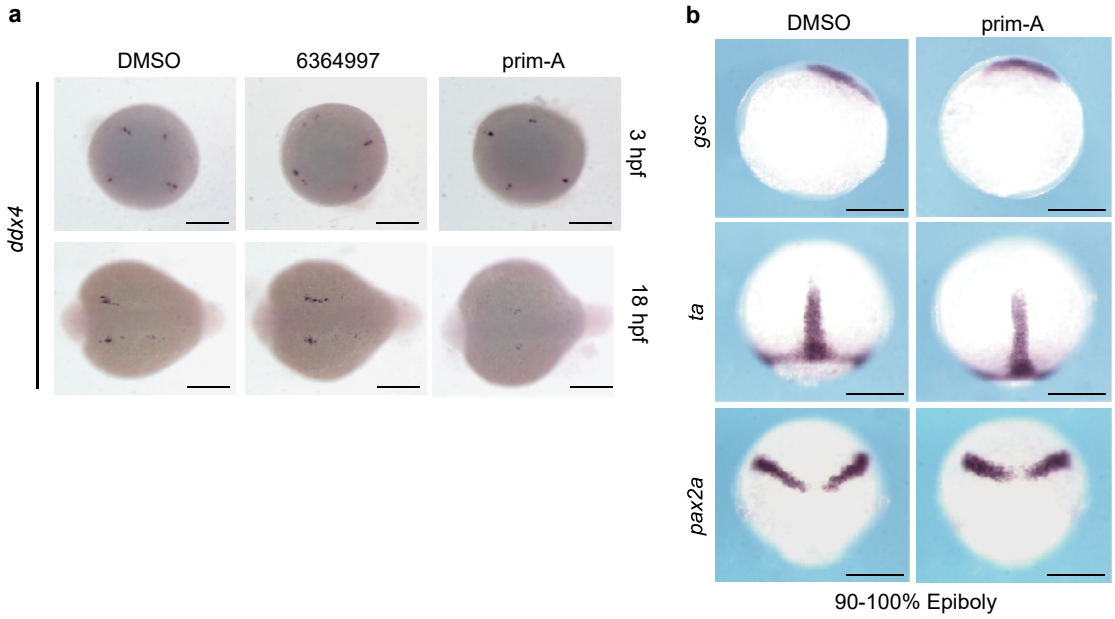
Supplementary Figure 2: Assessment of toxicity of primordazines. (**a**) Representative images of two embryos 24 h after treatment with different concentrations of prim-B. Prim-A treatment showed similar results (data not shown). This experiment was repeated two times with similar results. Scale bar = 500 μm. (**b**) Quantitative result shows that prim-A and –B do not exhibit any noticeable toxicity up to 10 μM. However, prim-A and –B at 15 μM led to a slight developmental delay. 20 μM treatment caused a noticeable toxicity in both prim-A and –B and 30 μM or higher led to a complete death. Overall, prim-B appears to be less toxic than prim-A. Developmental delay was defined as occurring when embryo shows more than 4 h of developmental delay (n = 2, independent experiments). Data is shown as mean ± s.d. (standard deviation).



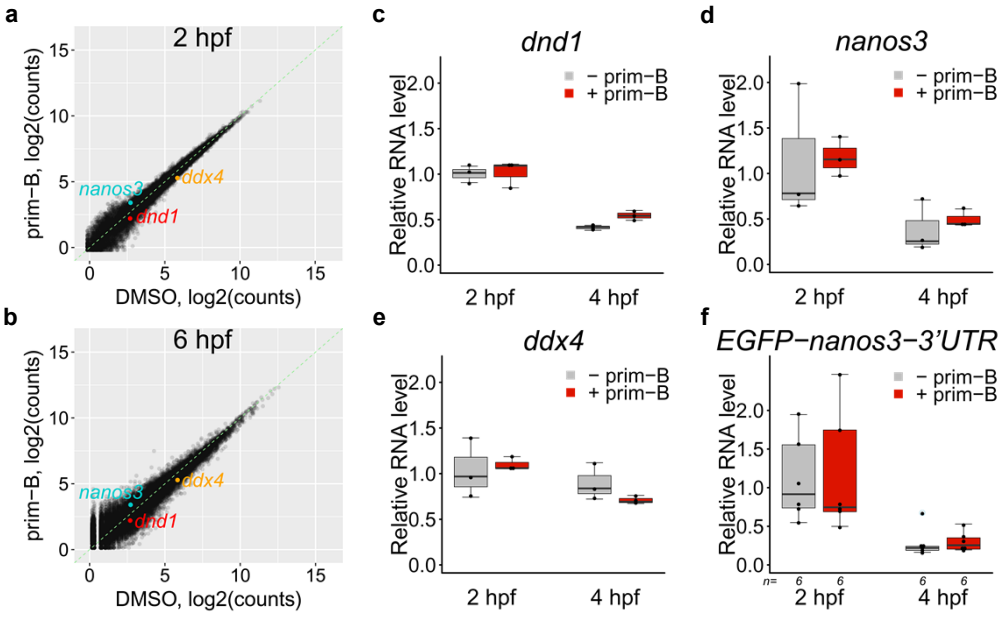
Supplementary Figure 3: Determination of a critical treatment time window for primordazine's activity on PGC loss. (**a**) *Tg(ddx4:EGFP)* embryos were treated with 10μ M prim-A for a variety of time courses, and EGFP fluorescence of PGCs was assessed visually. Primordazine requires treatment from 2 to 5 hpf in order to substantially decrease PGC number. (**b**) Injection of 2 mM prim-B at 1 hpf or at 6 hpf showed a different effect on PGC number at 24 hpf. 1-2 nl of 50% DMSO, or 2 mM prim-B in 50% DMSO was injected to yolk of embryos at 1 hpf or 6 hpf. Injection of prim-B at 1 hpf led to a significant reduction in PGC number as compared to DMSO, whereas injection of prim-B at 6 hpf did not change PGC number (*n* = number of animals). The box-and-whisker plot shows a median (centerline), upper/lower quartiles (box limits), maximum/minimum (upper/lower whiskers), and a light blue dot (outlier). Unpaired two-sided *t*-test. 95% confidence intervals.



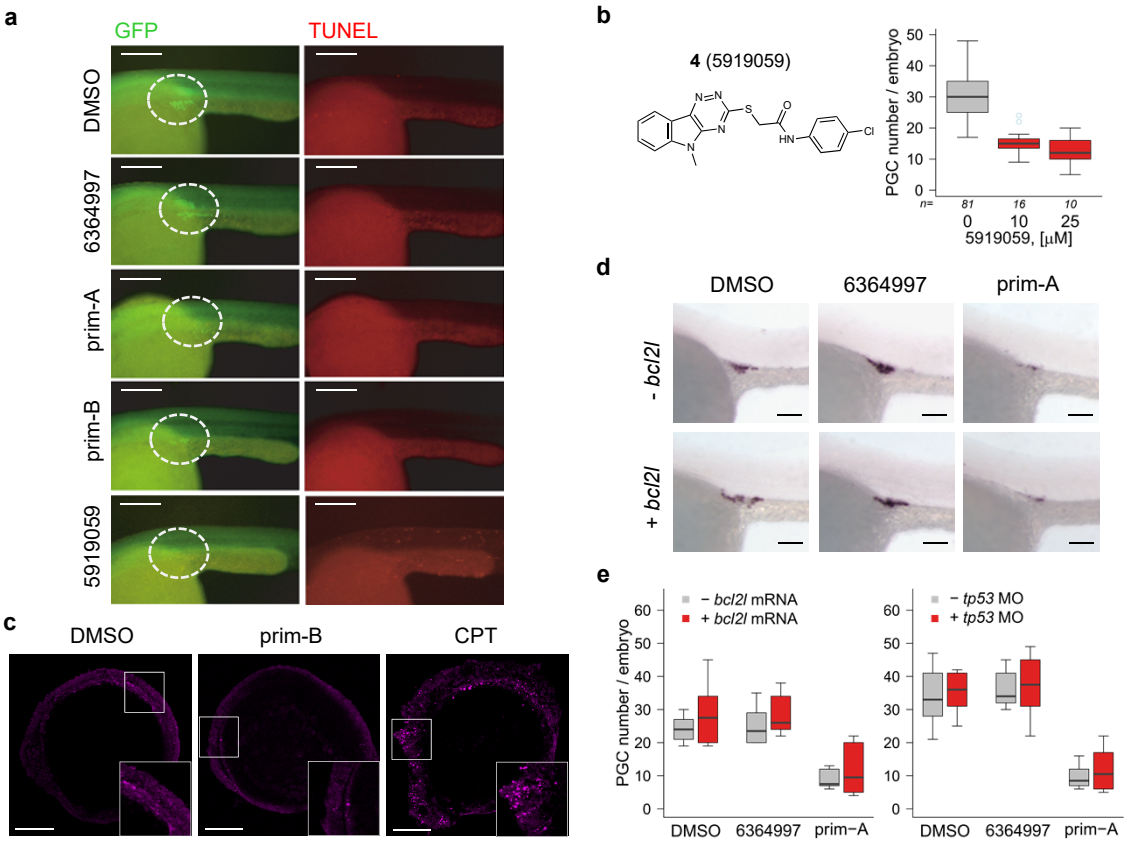
Supplementary Figure 4: Embryos treated with primordazine develop a skewed sex distribution to male. Embryos were treated with the indicated concentration of prim-B for 1 d and raised to adulthood. Sex was determined at 3 month old age. (*n* = 4 (148 fish), *n* = 3 (66 fish), *n* = 3 (77 fish), *n* = 3 (82 fish), and *n* = 4 (83 fish) for 0, 2.5, 5, 10, and 20 of prim-B (μM), respectively.) Data is shown as mean ± s.e.m. (standard error of mean).



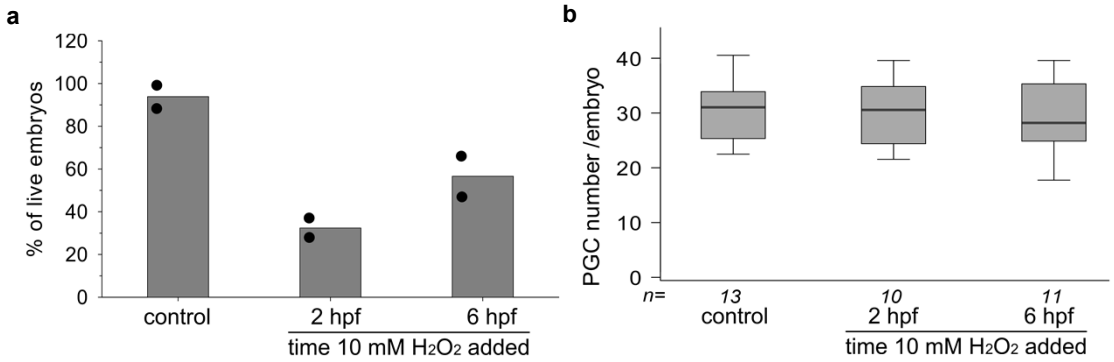
Supplementary Figure 5: Primordazine does not affect the specification or migration of PGCs. (**a**) WISH of *ddx4* does not show a noticeable change by prim-A treatment at the stage of specification (3 hpf) or migration (18 hpf) during PGC development, although PGC numbers are decreased at 18 hpf in response to prim-A. This experiment was repeated two times with similar results. Scale bar = 250 μm. (**b**) WISH for mesodermal markers (*gsc*, *ta*, and *pax2a*) indicates that prim-A does not affect the development of mesoderm of which a part will give rise to gonad. This experiment was repeated two times with similar results. Scale bar = 250 μm.



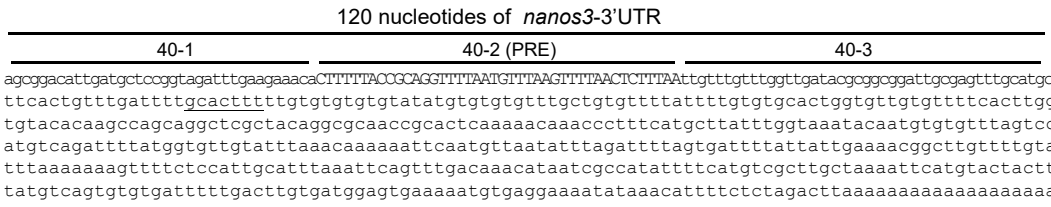
Supplementary Figure 6: Primordazine treatment does not change RNA levels. (**a**,**b**) RNA-seq analyses were performed from total RNA of embryos treated with DMSO or 10μM prim-B for the indicated period of time as described in the Methods. Graphs were generated by merging the two independent replicates. Overall RNA levels including *dnd1*, *nanos3*, and *ddx4* exhibit no difference between DMSO and prim-B conditions at (**a**) 2 hpf or (**b**) 6 hpf. (**c-f**) The qPCR for the levels of *dnd1* (**c**), *nanos3* (**d**), and *ddx4* (**e**) mRNAs at the different time points showed that prim-B treatment does not affect their RNA stability (*n* = 3, independent experiments). In addition, the level of *EGFP-nanos3-3'UTR* reporter mRNA (**f**) was not regulated by prim-B treatment. For **c-f**, box-and-whisker plots show a median (centerline), upper/lower quartiles (box limits), maximum/minimum (upper/lower whiskers), and a light blue dot (outlier).



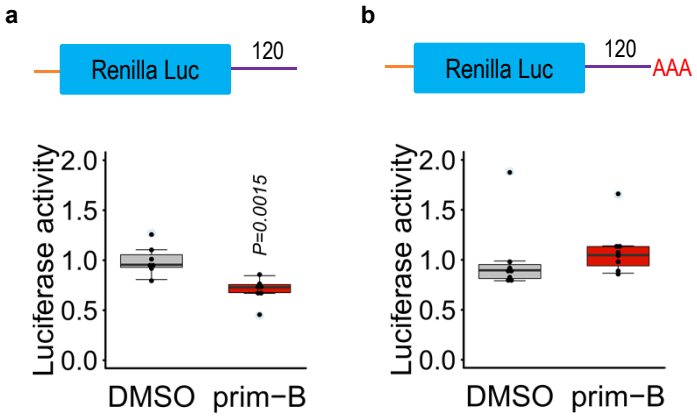
Supplementary Figure 7: Loss of PGCs by primordazine is not attributed to apoptosis. (**a**) Primordazine treatment does not induce apoptosis in the genital ridge or elsewhere in embryos at 24 hpf as measured by TUNEL staining, whereas 5919059, an active primordazine derivative, results in an increase of TUNEL positive cells predominantly outside of the genital ridge. The presumptive area for PGC location is marked by a white dotted line. Scale bar, 250 μm. (**b**) Treatment with 5919059 decreases PGC number (*n* = number of animals). (**c**) Immunostaining for activated caspase-3 does not detect the difference in apoptosis between DMSO and 10 μM prim-B treated embryos. Embryos were collected at 12 hpf. 100 nM of camptothecin (CPT) was treated from 8 hpf as a positive control. Scale bar, 200 μm. (**d**) Overexpression of an anti-apoptotic factor *bcl2-like* by injection of its mRNA prior to prim-A treatment does not rescue PGCs, as assessed by WISH for *ddx4*. Scale bar, 100 μm. (**e**) No effect of *bcl2l* on prim-A induced PGC loss was detected (*n* = 10). In addition, knockdown of pro-apoptotic factor *tp53* by antisense morpholino does not protect PGCs against prim-A (*n* = 10, number of animals). PGC numbers were counted as EGFP positive cells in *Tg(ddx4:EGFP)* embryos. Box-and-whisker plots in **b** and **e** show a median (centerline), upper/lower quartiles (box limits), maximum/minimum (upper/lower whiskers), and light blue dots (outliers). For **a**, **c**, and **d**, the experiment was repeated two times with similar results.



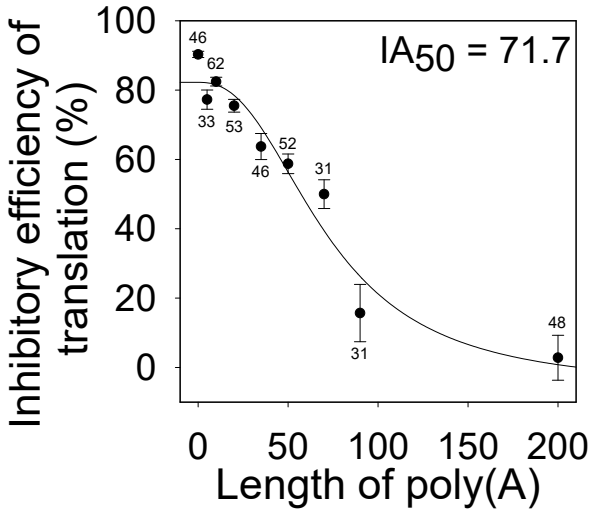
Supplementary Figure 8: PGCs are resistant to general toxicity. (**a**) Treatment of 10 mM H_2O_2 at different time points caused a severe toxicity, leading to a lower viability of embryos at 24 hpf (*n* = 2, independent experiments). Data is shown as mean ± s.d. (standard deviation). (**b**) The number of PGCs in embryos at 24 hpf was not affected after treatment with 10 mM H_2O_2. PGC numbers were counted as EGFP positive cells in *Tg(ddx4:EGFP)* embryos (*n* = number of animals). The box-and-whisker plot shows a median (centerline), upper/lower quartiles (box limits), and maximum/minimum (upper/ lower whiskers).



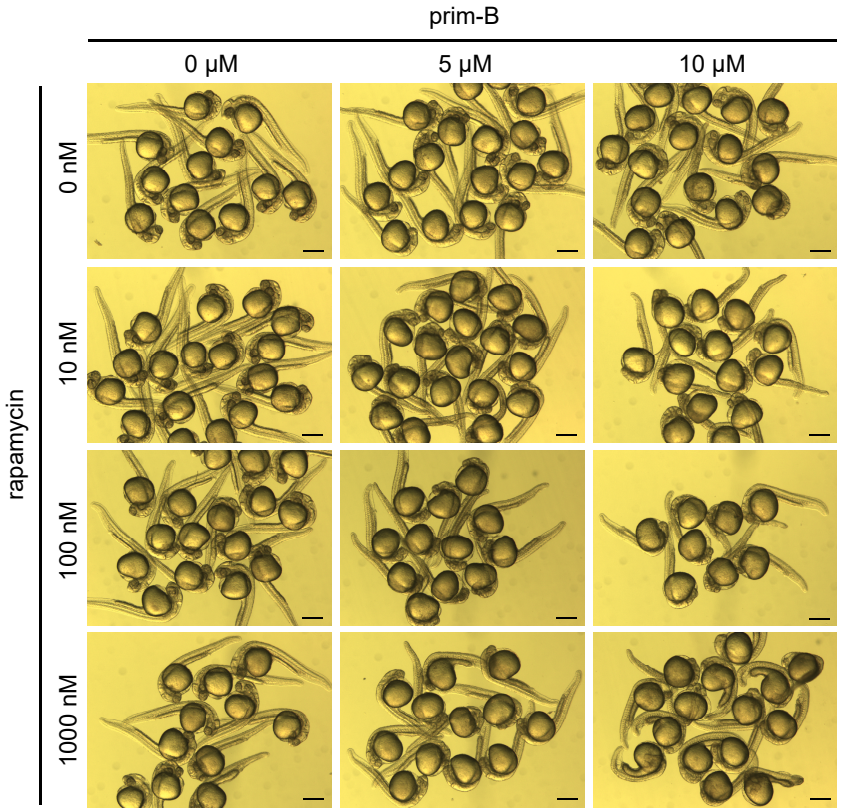
Supplementary Figure 9: The nucleotide sequence of the *nanos3*-3'UTR. The sequence of 120 nucleotides in the *nanos3*-3'UTR shows three regions consisting of 40-1, 40-2 (PRE), and 40-3 nucleotides. The seed site for miR-430 is indicated by a underline.



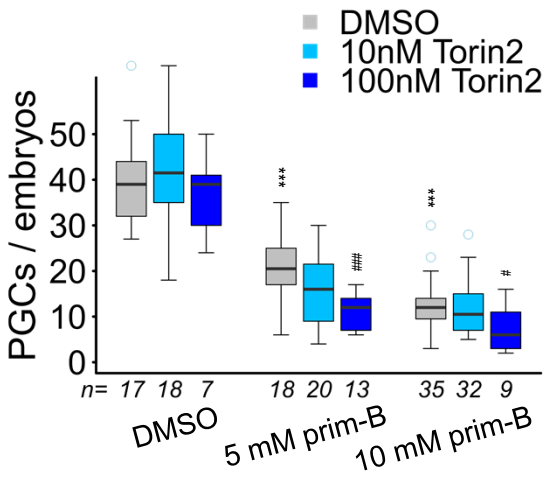
Supplementary Figure 10: The mechanism of primordazine's action does not involve the 5'-cap. (**a**) Primordazine represses the translation of luciferase mRNA with a PRE-120 independent of the 5'-cap (*n* = 7, independent experiments). (**b**) The refractory effect of polyadenylation on primordazine-induced translational inhibition does not require a 5'-cap (*n* = 8, independent experiments). Unpaired two-sided t-test with 95% confidence intervals. Box-and-whisker plots show a median (centerline), upper/lower quartiles (box limits), maximum/minimum (upper/lower whiskers), and light blue dots (outliers).



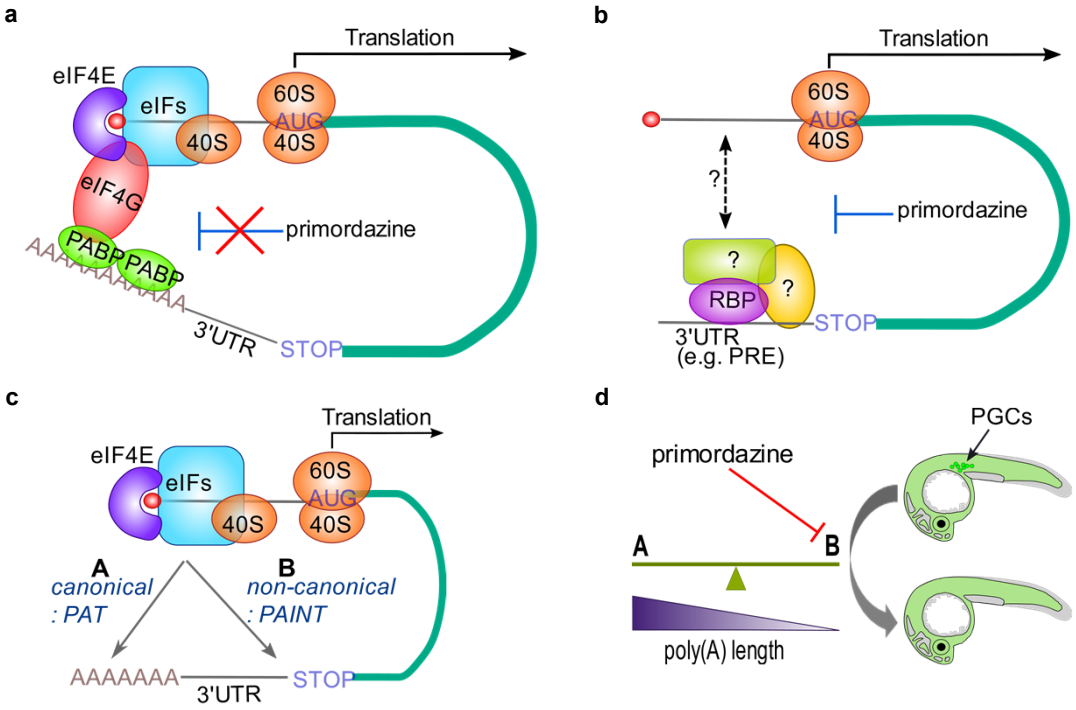
Supplementary Figure 11: Inhibitory activity of prim-B on translation was inversely correlated with poly(A)-tail length. A series of EGFP-120 constructs possessing differing poly(A)-tail lengths were made and *in vitro* transcribed. mRNAs from each construct were injected into zebrafish embryos. GFP fluorescence was measured and inhibitory efficiency of prim-B on translation was calculated. The number of adenosines enabling 50% translational inhibition by primordazine (IA_{50}) was determined to be 72. The number next to each data point indicates the sample size (*n*) (*n* = animals per condition). Data is represented as mean ± s.e.m..



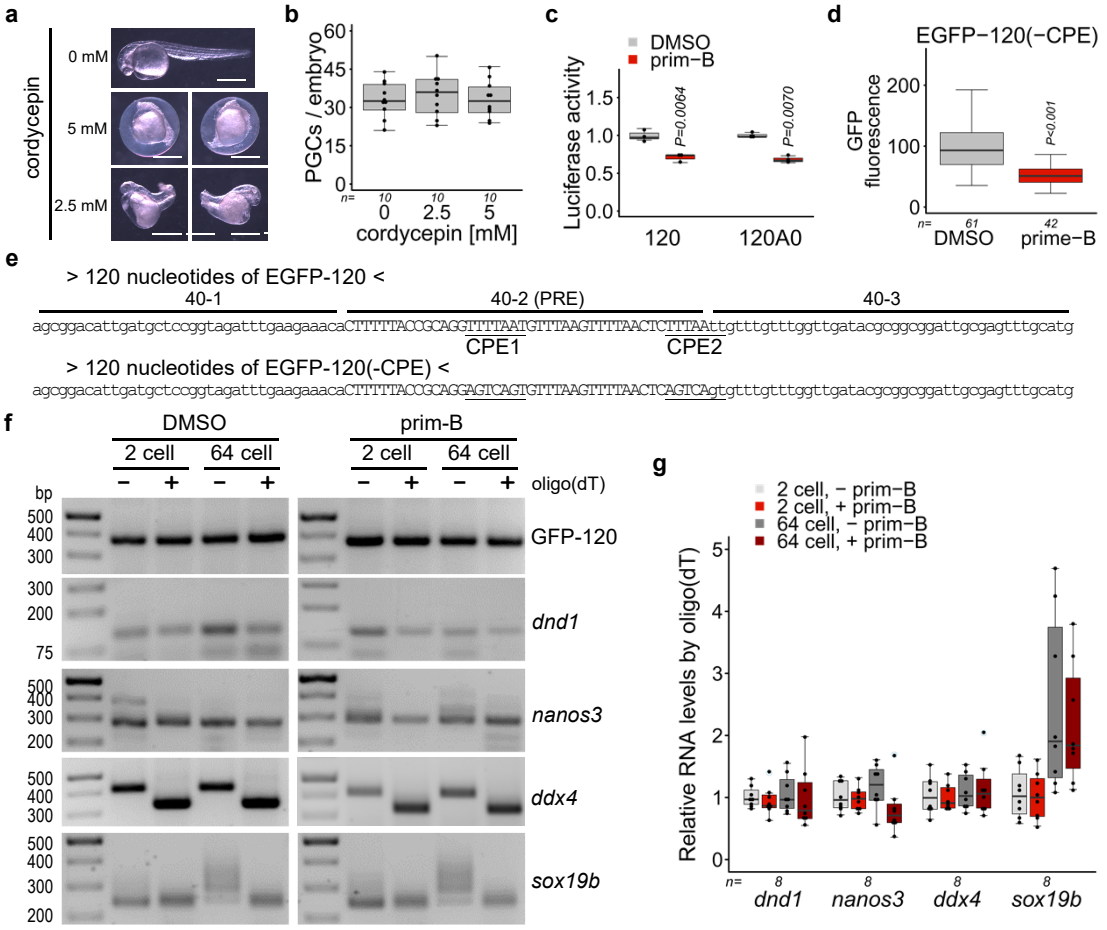
Supplementary Figure 12: Treatment of rapamycin or rapamycin and prim-B does not cause a significant toxicity. Embryos were treated prior to 1 hpf at different concentrations of prim-B, rapamycin, or both. No difference in the viability at 24 hpf was observed among all conditions (data not shown). Embryos cotreated with 1000 nM , and 5 µM or 10 µM prim-B exhibit a mild developmental delay. This experiment was repeated two times with similar results. Scale bar = 500 µm.



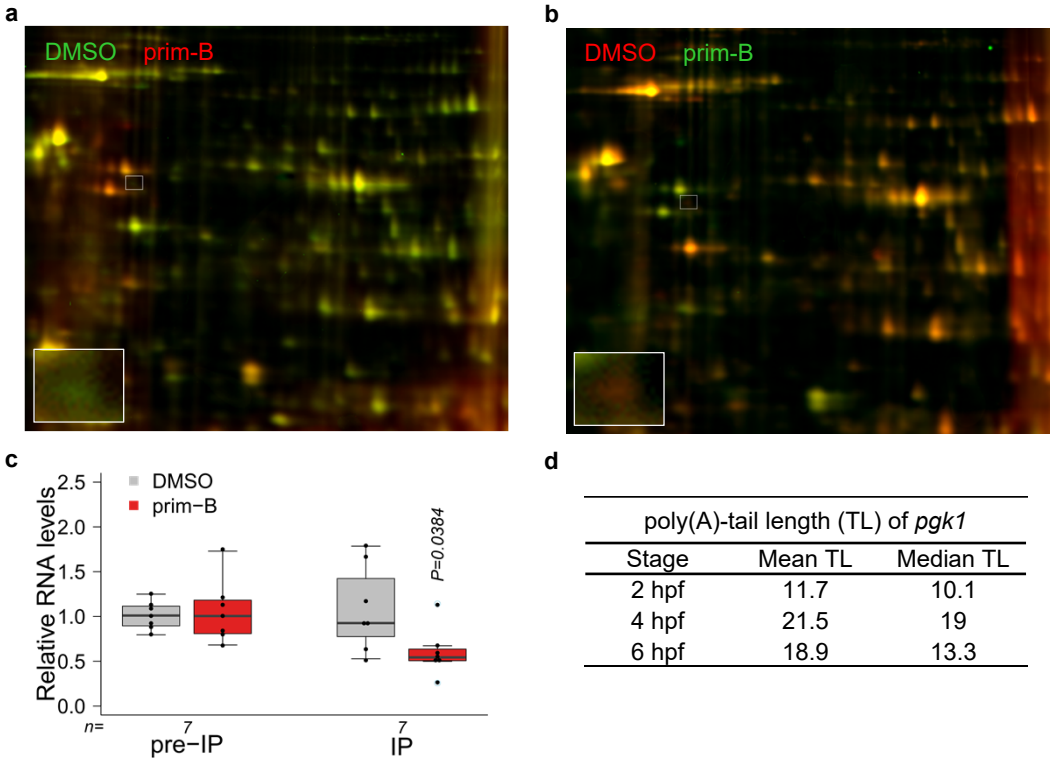
Supplementary Figure 13: Synergistic effect of torin2 on primordazine-mediated PGC loss. A direct mTOR inhibitor, torin2, potentiates primordazine in reducing PGC number (*n* = independent experiments). *** *vs.* DMSO; #,### *vs.* prim-B; # $P<0.05$; ***,### $P<0.001$. Unpaired two-sided *t*-test with 95% confidence intervals. Box-and-whisker plots show a median (centerline), upper/lower quartiles (box limits), maximum/minimum (upper/lower whiskers), and light blue dots (outliers).



Supplementary Figure 14: Models of canonical and non-canonical translation. (**a**) Model of poly(A)-tail dependent canonical translation (PAT). PABP binds to the poly(A)-tail and this interaction allows eIF4G to interact with PABP, leading to association of eIF4G with eIF4E bound to the 5'-cap. This coordinated interactions result in a closed-loop formation that allows for translation initiation and is not inhibited by primordazine. (**b**) Proposed model of poly(A)-tail independent non-canonical translation (PAINT). Unlike PAT, mRNA with a very short or no poly(A)-tail may recruits RNA-binding protein(s) (RBPs) to their specific motif such as PRE in the 3'UTR, which in turns recruits additional unidentified factors that play an important role in translation initiation for PAINT. Unlike the closed-loop formed by PAT, primordazine disrupts the function of unidentified factor(s) that plays an important role in the closed-loop formation in PAINT, leading to translation inhibition. In both models, mRNA may be circularized by eIF4G or other less-canonical factor(s) such as eIF3. (**c**) A proposed model of translation in the early embryos. Translation during early embryogenesis takes place via canonical or non-canonical pathways. The canonical pathway involves a poly(A)-tail while the non-canonical pathway occurs via a deadenylated 3'UTR. (**d**) The length of a poly(A)-tail determines the translational direction by competition between canonical and non-canonical pathways. Primordazine only inhibits non-canonical translation in the early embryos, PAINT, leading to loss of PGC.



Supplementary Figure 15: Cytoplasmic polyadenylation is not involved in primordazine's mechanism of action. (**a**) Cordycepin causes severe developmental defects. 1-2 nl of cordycepin at 2.5 mM or 5 mM was injected into yolk of embryos prior to 1 hpf. Representative images of embryos at 24 hpf are shown. This experiment was repeated two times with similar results. Scale bar = 500 μm. (**b**) PGC numbers were not changed by cordycepin injection (*n* = number of animals). (**c**) Prim-B reduces translation of luciferase mRNA containing 120 PRE in the 3'UTR ending A0, which is cordycepin, to a similar degree as shown in the same mRNA without A0. (*n* = 3, independent experiments). Unpaired two-sided *t*-test. 95% confidence intervals. (**d**) Prim-B treatment reduces GFP fluorescence of EGFP-120(-CPE) mRNA which has mutations at two putative CPE sites (*n* = number of animals). Unpaired two-sided *t*-test. 95% confidence intervals. (**e**) The sequence of 120 nucleotides in the *nanos3*-3'UTR shows two putative CPEs, CPE1 and CPE2. Two CPE sites were mutated in EGFP-120(-CPE). (**f**) RNA-ligation mediated poly(A) test (RL-PAT) showed that cytoplasmic polyadenylation is unlikely a contributing factor for the poly(A)-tail length in EGFP-120, *dnd1*, *nanos3*, and *ddx4* mRNA. In contrast, sox19b mRNA undergoes cytoplasmic polyadenylation, resulting in a significant increase in the length of poly(A)-tail. This experiment was repeated two times with similar results. (**g**) The real time-PCR using cDNA synthesized with oligo(dT) was performed to assess polyadenylation between 2 cell and 64 cell stages in the presence or absence of prim-B. The result shows that cytoplasmic polyadenylation does not occur in *dnd1*, *nanos3*, and *ddx4* mRNAs, while *sox19b* mRNA exhibits a significant increase in polyadenylation at 64 cell stage. Importantly, prim-B does not affect cytoplasmic polyadenylation (*n* = independent experiments). For **b**, **c**, **d**, and **g**, box-and-whisker plots show a median (centerline), upper/lower quartiles (box limits), maximum/minimum (upper/lower whiskers), and light blue dots (outliers).

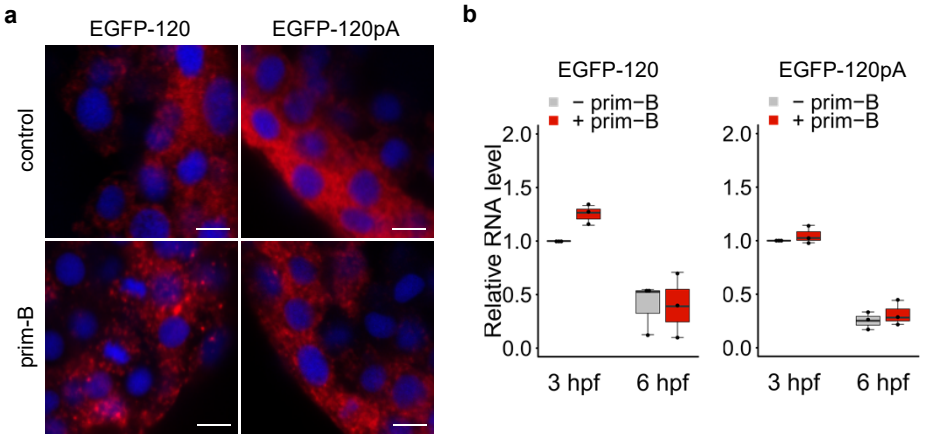


poly(A)-tail length (TL) of *pgk1*

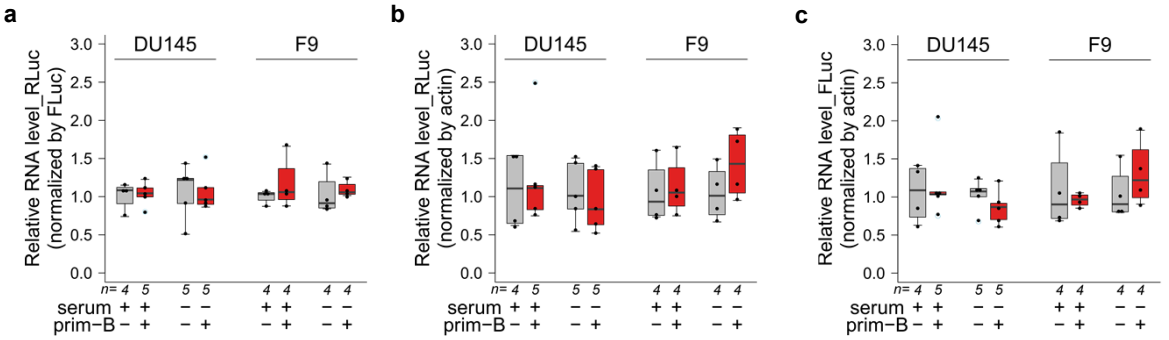
Stage	Mean TL	Median TL
2 hpf	11.7	10.1
4 hpf	21.5	19
6 hpf	18.9	13.3

Supplementary Figure 16: Primordazine specifically inhibits translation of endogenous gene containing a short poly(A)-tail. (**a,b**) 2D-DIGE was performed to identify proteins whose expression is altered by prim-B treatment. The same amount of protein from each condition was labeled by Cy3 or Cy5 dye, combined, and processed for 2D-DIGE. To minimize experimental variations, color was swapped in independent experiments. Color codes indicate sample conditions shown in corresponding colors. The white box contains a spot identified as *pgk1*. The inset shows the enlarged spot which exhibits green (**a**) or red (**b**) fluorescence due to translation inhibition by prim-B treatment. This experiment was repeated three times with similar results. (**c**) TRAP result shows that translation but not RNA levels of *pgk1* is inhibited by prim-B treatment (*n* = independent experiments). The box-and-whisker plots show a median (centerline), upper/lower quartiles (box limits), maximum/minimum (upper/lower whiskers), and light blue dots (outliers). Unpaired two-sided *t*-test. 95% confidence intervals. (**d**) The poly(A)-tail lengths of *pgk1* was obtained from the previously published data (Subtelny et al[1]). The poly(A)-tails of *pgk1* mRNAs remain very short up to 6 hpf.

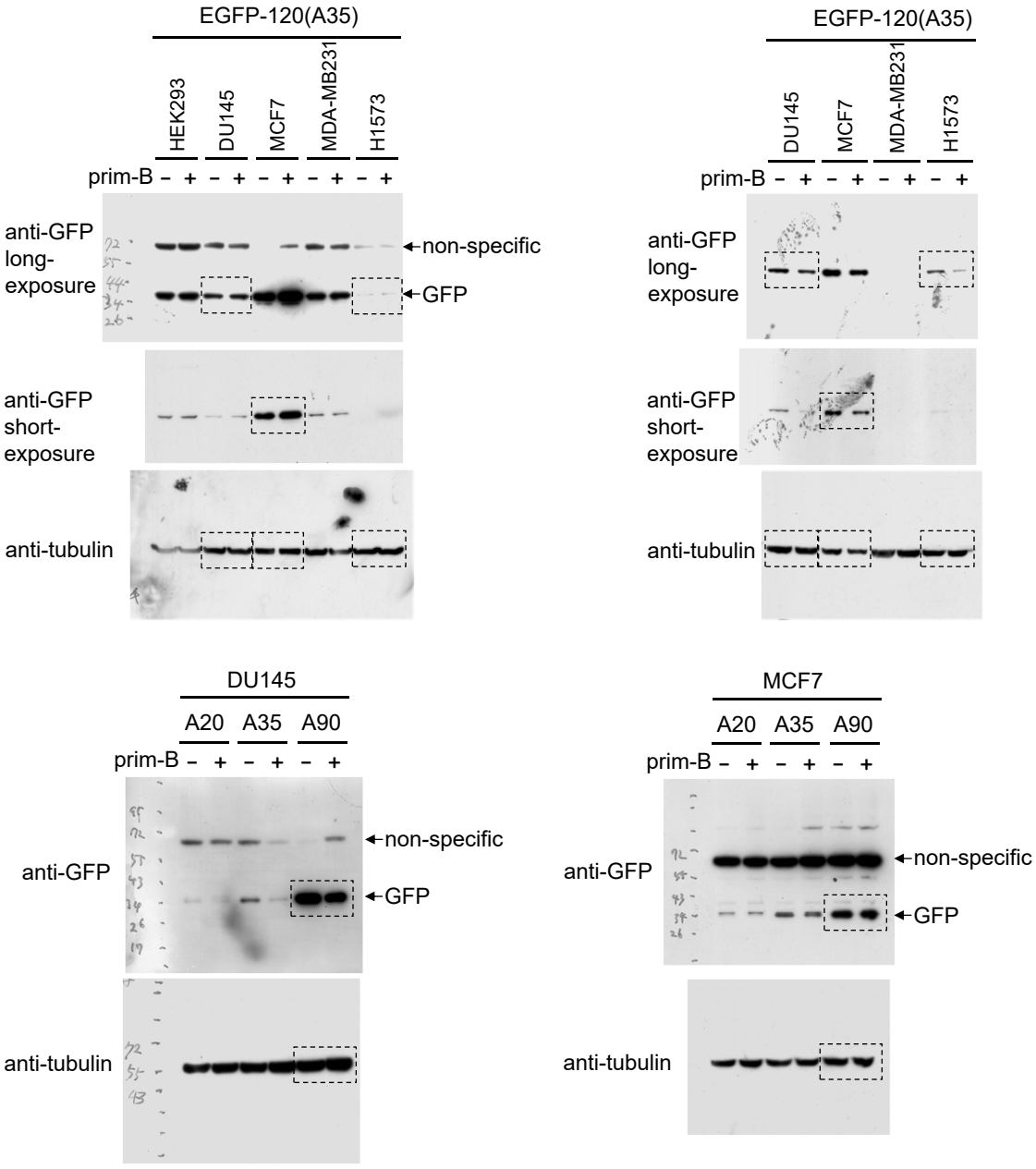
1. Subtelny, A. O., Eichhorn, S. W., Chen, G. R., Sive, H. & Bartel, D. P. Poly(A)-tail profiling reveals an embryonic switch in translational control. *Nature* **508,** 66–71 (2014).



Supplementary Figure 17: Primordazine induces the formation of abnormal granules of exogenous mRNA without a poly(A)-tail. (**a**) Equal amounts of EGFP-120 or EGFP-120pA mRNAs were injected followed by staining of EGFP mRNA at 8 hpf. Prim-B increases the level of noticeable RNA granules in EGFP-120, whereas EGFP-120pA mRNAs do not display granule formation. Scale bar, 10 μm. This experiment was repeated two times with similar results.(**b**) The qPCR for the levels of EGFP-120 or EGFP-120pA mRNAs at the different time points revealed transient stabilization of EGFP-120 mRNAs by prim-B treatment at 3 hpf, while the degradation rate of EGFP-120pA mRNAs was not affected by prim-B (*n* = 3, independent experiments). Box-and-whisker plots show a median (centerline), upper/lower quartiles (box limits), and maximum/minimum (upper/lower whiskers).



Supplementary Figure 18: Primordazine treatment does not change RNA levels in mammalian cells. (**a**) The qPCR results showed that prim-B treatment did not affect RNA levels of RLuc-120 in the presence or absence of serum in the two mammalian cell lines DU145 and F9. RNA levels of RLuc-120 were normalized by FLuc-SV40pA mRNA. (**b**) RLuc-120 and (**c**) FLuc-SV40pA RNA levels were normalized by *actin* mRNA. Prim-B treatment did not show a noticeable change in the RNA levels of RLuc-120 or FLuc-SV40pA in the presence or absence of serum in both cell types. *n* = independent experiments. Box-and-whisker plots show a median (centerline), upper/lower quartiles (box limits), maximum/minimum (upper/lower whiskers), and light blue dots (outliers).



Supplementary Figure 19: Full images of Western blots displayed in the Fig. 6a.

Supplementary Table 1. Small molecule screening data

Category	Parameter	Description
Assay	Type of assay	In vivo phenotypic screen
	Target	Primordial germ cells (PGCs)
	Primary measurement	GFP fluorescence
	Key reagents	primordazine
	Assay protocol	Approximately 5 – 10 *Tg(vasa/ddx4:EGFP)* embryos expressing EGFP in PGCs were placed per well in 96 well plates. 7,000 small molecules from the ChemBridge DiverSet library were added to each well at a concentration of ~ 10 μM prior to 1 hpf. The number of PGCs in embryos at 22-24 hpf was visually assessed by EGFP fluorescence using a fluorescence stereomicroscope.
	Additional comments	
Library	Library size	7,000 small molecules
	Library composition	
	Source	ChemBridge DiverSet library
	Additional comments	
Screen	Format	96-well plates
	Concentration(s) tested	~ 10 μM
	Plate controls	DMSO
	Reagent/ compound dispensing system	Multichannel pipette
	Detection instrument and software	Zeiss fluorescent stereomicroscope
	Assay validation/QC	
	Correction factors	
	Normalization	
	Additional comments	
Post-HTS analysis	Hit criteria	Reduction in PGC number to <10 PGCs/embryo
	Hit rate	2/7000 = 0.029%
	Additional assay(s)	
	Confirmation of hit purity and structure	Mass spectrometry, HPLC and NMR
	Additional comments	