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SMAUG Is a Major Regulator of Maternal mRNA Destabilization in *Drosophila* and Its Translation Is Activated by the PAN GU Kinase

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SUMMARY

In animals, egg activation triggers a cascade of posttranscriptional events that act on maternally synthesized RNAs. We show that, in *Drosophila*, the PAN GU (PNG) kinase sits near the top of this cascade, triggering translation of SMAUG (SMG), a multifunctional posttranscriptional regulator conserved from yeast to humans. Although PNG is required for cytoplasmic polyadenylation of *smg* mRNA, it regulates translation via mechanisms that are independent of its effects on the poly(A) tail. Analyses of mutants suggest that PNG relieves translational repression by PUMILIO (PUM) and one or more additional factors, which act in parallel through the *smg* mRNA's 3′ untranslated region (UTR). Microarray-based gene expression profiling shows that SMG is a major regulator of maternal transcript destabilization. SMG-dependent mRNAs are enriched for gene ontology annotations for function in the cell cycle, suggesting a possible causal relationship between failure to eliminate these transcripts and the cell cycle defects in *smg* mutants.

INTRODUCTION

Early metazoan development is controlled by maternally loaded RNAs and proteins. Whereas maternal transcripts are stable in mature oocytes, egg activation triggers destabilization of a subset of the maternal mRNAs (reviewed in Tadros and Lipshitz, 2005). In *Drosophila*, these include *Hsp83*, *nanos*, *string*, *Pgc*, and *cyclin B* mRNA (Bashirullah et al., 1999, 2001; Edgar and Datar, 1996; Semotok et al., 2005; Tadros et al., 2003). Degradation is accomplished through the joint action of two pathways that eliminate transcripts by interphase of nuclear division cycle 14, 2 to 3 hours after fertilization. Elimination of these transcripts has been hypothesized to terminate maternal control of embryogenesis and enable patterned zygotic transcription to assume command.

An important factor in transcript destabilization, SMAUG (SMG), was first identified as a protein that represses translation of *nanos* mRNA in the bulk cytoplasm of the early embryo by binding to 3′ untranslated region (UTR) elements known as SMG response elements (SREs) (Dahanukar et al., 1999; Smibert et al., 1999) and recruiting the eIF-4E binding protein, CUP (Nelson et al., 2004). Subsequently, it was found that *Hsp83* mRNA is targeted for degradation by SMG (Semotok et al., 2005). Although SMG does not translationally repress maternal *Hsp83* transcripts, it is essential for their destabilization, which it triggers by recruiting the CCR4/POP2/NOT deadenylase in an SRE-independent manner. Thus, SMG is a multifunctional posttranscriptional regulator that controls both maternal transcript stability and translation.

To carry out a global search for SMG's targets, we have used microarray-based gene expression profiling. Our results show that over half of the genome is represented in maternal mRNAs; that more than a fifth of these are destabilized as a result of egg activation; and that two thirds of the unstable mRNAs depend on SMG for degradation. Thus, SMG is a major regulator of maternal transcript destabilization upon egg activation.

Embryos produced by *smg* mutant females exhibit cell cycle defects beginning at the eleventh syncytial nuclear

division and cease development prior to the midblastula transition (MBT) (Dahanukar et al., 1999). We show that SMG-dependent transcripts are enriched for gene ontology (GO) terms related to mitosis and the cell cycle. In light of the *smg* mutant's cell cycle defects, our data suggest that normal progression through the late syncytial divisions requires destabilization of maternal cell cycle mRNAs.

SMG synthesis is, itself, a consequence of posttranscriptional control: although *smg* mRNA is expressed maternally, SMG protein is absent from mature oocytes but is present in embryos (Dahanukar et al., 1999; Smibert et al., 1999). Here we show that the PAN GU (PNG) kinase complex is required following egg activation for the translation of *smg* mRNA, thus linking the signal provided by egg activation with the posttranscriptional regulation of maternal transcripts. This explains our recovery of mutations in genes encoding components of this complex—*pan gu* (*png*), *plutonium* (*plu*), and *giant nuclei* (*gnu*)—in a previous search for destabilization-defective maternal effect mutants (Tadros et al., 2003). Prior to that screen, PNG had been identified as a coordinator of the early embryonic cell cycle through maintenance of mitotic Cyclin levels (Elfring et al., 1997; Fenger et al., 2000; Lee et al., 2001, 2003; Renault et al., 2003; Shamanski and Orr-Weaver, 1991). However, though restoring Cyclins to normal levels in *png* mutants rescues the cell cycle (Lee et al., 2001), it does not rescue transcript destabilization (Tadros et al., 2003). From these and other genetic experiments, we concluded that the PNG complex independently regulates the cell cycle and maternal transcript stability. PNG's control of both *smg* (this study) and *cyclin B* (Vardy and Orr-Weaver, 2007, published in this issue of *Developmental Cell*) translation explains the above duality of its biological functions.

We demonstrate that, although PNG is required for the cytoplasmic polyadenylation of *smg* mRNA, restoration of polyadenylation in *png* mutants is not sufficient to rescue SMG translation. Thus, PNG regulates the translation of *smg* mRNA via mechanisms that are independent of its effects on polyadenylation. It has recently been reported that *smg* mRNA is in a complex with PUMILIO (PUM) protein (Gerber et al., 2006). We present evidence consistent with the hypothesis that, upon egg activation, PNG relieves repression by PUM and one or more parallel-acting translational repressors. These results complement those of Vardy and Orr-Weaver (2007) showing that removal of the PUM repressor in *png*; *pum* double mutants restores translation of *cyclin B* mRNA.

Our data place the PNG kinase near the top of the major posttranscriptional regulatory cascade that triggers maternal RNA destabilization and progression through the syncytial nuclear divisions.

RESULTS

Egg Activation Triggers Destabilization of Over 1600 Maternal mRNAs

To carry out global analyses of SMG's role in maternal mRNA destabilization, we first needed to identify all maternal mRNAs and assess their stability in wild-type. *Drosophila* stage 14 oocytes served as our reference as they are fully loaded with maternal transcripts and protein, and are poised for activation and fertilization upon passage into the oviducts and the uterus, respectively. Gene expression profiling led us to calculate that approximately 55% of the genome is present in these mature oocytes (see Experimental Procedures). To define the fraction of maternal mRNAs that is destabilized upon egg activation we followed 5097 maternal mRNAs during the first 6 hours after egg activation (Figure 1A). Consistent with the fact that unfertilized eggs are transcriptionally silent, the number of upregulated transcripts observed was within the limits of expected experimental variability: 1% showed a 1.5-fold and 0.02% showed a 2-fold or greater increase. In contrast, 21% of transcripts (1069) showed a 1.5-fold or greater decrease and half of these were reduced by at least 2-fold. Forty-five transcripts decreased at least 5-fold in abundance, including *Hsp83*, previously identified as a target for destabilization by SMG (Semotok et al., 2005). The observed decreases are biased toward abundant mRNAs (R = 0.56), consistent with the fact that rare transcripts reach background levels after undergoing a smaller fold reduction than abundant transcripts. Extrapolating to the whole genome, we estimate that over 1600 of 7745 maternally deposited mRNAs are destabilized upon egg activation.

Two Thirds of the Unstable Maternal Transcripts Are SMG Dependent

To assess the role of SMG in maternal transcript destabilization, we profiled transcripts in activated eggs produced by *smg* mutant females. RNA populations in *smg* versus wild-type stage 14 oocytes showed over 99% overlap, confirming that SMG does not act prior to egg activation. Strikingly, two thirds of the transcripts (712 of 1069) scored as unstable in wild-type were stabilized in *smg* mutant activated eggs (Figures 1B and 1C). Thus, SMG is a major regulator of maternal transcript destabilization in activated eggs.

Computational Analysis of the Transcript Classes

We analyzed the different classes of transcripts for 3′ UTR length, SREs, micro-RNA (miRNA) binding sites, and GO terms. Consistent with the fact that posttranscriptional regulation often uses *cis* elements in the 3′ UTR, the mean length of maternal transcript 3′ UTRs is 1.5-fold greater than that of nonmaternal transcripts: 421 nucleotides (nt) versus 279 nt, respectively. All subsequent calculations were normalized to UTR length.

Unstable maternal mRNAs are enriched for SREs when compared with stable mRNAs: 2-fold enrichment in open reading frames (ORFs) (135 SREs per megabase [Mb] in unstable versus 72 in stable) and 1.5-fold enrichment in 3′ UTRs (31 SREs/Mb in unstable versus 20 in stable). Higher GC content is likely to be the cause of the increased SRE counts within coding regions. The SMG-dependent and SMG-independent unstable mRNAs were not significantly different in SRE enrichment (ORFs: 131

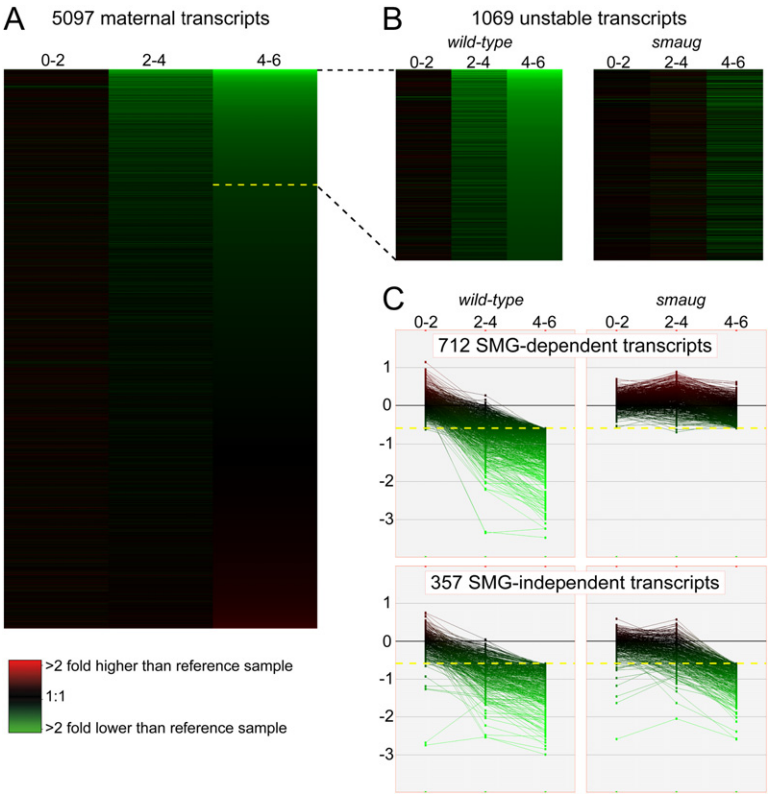

Figure 1. Microarray-Based Gene Expression Profiling of Maternal Transcript Stability in Activated Unfertilized Eggs from Wild-Type and *smg* Mutant Females

Unfertilized eggs were collected 0–2, 2–4, and 4–6 hr after laying.

(A) Maternal mRNAs (5097) sorted according to instability at 4–6 hr; each is represented by a horizontal bar, with black indicating no change, green a decrease, and red an increase in transcript abundance relative to stage 14 oocytes. Transcripts above the dashed yellow line (ratio's log base 2 of −0.59 = 1.5-fold decrease) are significantly destabilized.

(B) Left: the 1069 transcripts that are significantly destabilized in wild-type. Right: the same transcripts in eggs from *smg* mutant females, showing that many of these are stabilized.

(C) Unstable transcripts can be subdivided into two classes. Upper: 712 SMG-dependent transcripts. Lower: 357 SMG-independent transcripts. Y axis: ratio's log base 2; dashed yellow line as in (A).

SREs/Mb in SMG-dependent versus 145 in SMG-independent; 3′ UTRs: 30 SREs/Mb in SMG-dependent versus 31 in SMG-independent). Restricting analyses to SREs that occur only within evolutionarily conserved regions did not affect the outcome.

The average number of 3′ UTR targets per miRNA was 84, with a range of 6 (*miR-100*) to 544 (*miR-289*). Maternal transcript 3′ UTRs were significantly enriched for target sites for 11 miRNAs (see Figure S1 and Table S1 available in the Supplemental Data available with this article online). Most of these enriched miRNAs are present during the first several hours of embryogenesis (Aravin et al., 2003; Leaman et al., 2005), the stages during which maternal mRNAs control embryonic development. *miR-289* and *miR-277* target sites were most highly enriched and the top GO biological process "hits" (Ashburner et al., 2000) for their targets were "morphogenesis" and "development."

To assess GO term enrichment, we used EASE analysis (Hosack et al., 2003). The most enriched GO term molecular category among stable transcripts was "structural constituent of the ribosome" (93/99 maternal ribosome constituent mRNAs are stable; EASE score of 2.35×10^{-5}), consistent with previous analyses which used *rpA1* and *rp49* as stable control mRNAs (Bashirullah et al., 1999; Semotok et al., 2005; Tadros et al., 2003). Enriched biological categories included "cell-cell communication," "RNA metabolism," "RNA binding," and "RNA splicing." For unstable mRNAs, "cell cycle" categories were enriched, representing four of the top ten categories (EASE scores from 6.21×10^{-7} to 3.8×10^{-4}). Other top-ten categories included "nuclear organization" and "chromosome organization and biogenesis."

SMG-independent unstable transcripts were enriched for GO terms related to "female gamete generation" and "oogenesis" (EASE scores of 0.036–0.049; Tables S2 and S3). Such transcripts include *germ cell-less*, *oskar*, *exuperantia*, *yolk protein 1*, *2*, and *3*, *ovarian tumor* (*otu*), *fs(1)Nasrat*, *orb*, *diminutive*, *yolkless*, *Jun-related antigen* (*Jra*), *puckered*, *shark*, *POSH*, *moira*, *Hsf*, *transformer 2*, *stem-loop binding protein*, *hunchback*, *Me31B*, and *bicoid stability factor*.

Cell cycle categories were further enriched in the SMG-dependent class: three of the five enriched categories were "cell cycle," "cell proliferation," and "DNA replication/chromosome cycle" (EASE scores of 0.016–0.030; Tables S2 and S3). Cell cycle-related transcripts include *aubergine*, *deadhead*, *loki*, *arrest* (*Bruno*), *twins*, *minispindles*, *pelota*, *Cks*, *fs(1)Ya*, *grapes*, *CKIIα*, *Rbf*, *Rbf2*, *Rab5*, *pavarotti*, *cyclins A* and *C*, *mei-P26*, *Cdc27*, and *bifocal*. The remaining two enriched categories relate to "protein or macromolecule catabolism" (EASE scores of 0.012 and 0.046) and include *CCR4*, *Nedd4*, *Ubiquitin activating enzyme 1*, and *rhomboid* mRNAs. Other SMG-dependent transcripts include *Hsp83*, *Hsp27*, *DNAJ-like*, *vasa*, *bicoid-interacting protein 3*, and *eIF-4G*. The enrichment of cell cycle-related mRNAs in the SMG-dependent class of unstable transcripts suggests a possible explanation for the *smg* mutant phenotype (see Discussion).

Mutants in the PNG Kinase Complex Lack SMG Protein

Because *smg* mRNA is present in both oocytes and early embryos but SMG protein is present only in the latter (Dahanukar et al., 1999; Smibert et al., 1999), *smg* mRNA is itself a candidate for posttranscriptional regulation. Having established that the PNG kinase complex is essential for maternal transcript degradation (Tadros et al., 2003), we asked whether SMG protein is present in *png*, *plu*, or *gnu* mutants. Strikingly, early embryos from *png*, *plu*, and *gnu* mutant mothers lacked detectable SMG protein (Figures 2 and 3C).

Unlike SMG, the PNG, PLU, and GNU proteins are present in both ovaries and embryos (Elfring et al., 1997; Fenger et al., 2000; Renault et al., 2003). We therefore asked whether the absence of SMG protein and the failure to destabilize maternal mRNAs in these mutants is a secondary consequence of defects during oogenesis. To do so, we engineered a situation in which functional PNG protein was absent during oogenesis and was synthesized upon egg activation: we produced flies carrying a transgene—henceforth called *bcd5′UTR-png-bcd3′UTR*—in which the *png* ORF is placed within the context of a genomic fragment that includes all of the endogenous *cis* elements that regulate *bicoid* (*bcd*) expression (Figure 3A). The *bcd* 3′ UTR restricts translation to embryos (Zhang et al., 2004). In a png^{158} background, *bcd5′UTR-png-bcd3′UTR* is the only source of full-length PNG protein, which was present in early embryos but not mature, stage 14 oocytes (Figure 3B). SMG protein accumulated and maternal *Hsp83* degradation was rescued in the anterior of *png* mutant mothers that carried *bcd5′UTR-png-bcd3′UTR* (Figure 3C). Thus, expression of PNG after egg activation is sufficient to restore SMG protein and to trigger maternal mRNA destabilization (PNG is synthesized only in the anterior because RNA localization elements present in the *bcd* 3′ UTR restrict *bcd5′UTR-png-bcd3′UTR* mRNA to the anterior pole).

PNG Is Required for SMG Translation and Acts through the *smg* mRNA 3′ UTR

PNG could function to control SMG expression by regulating *smg* transcription, mRNA stability, translation, and/or SMG protein stability. The first two possibilities were excluded because *smg* mRNA levels are not reduced in *png* embryos relative to wild-type (Figure 4A). To distinguish a role in translation versus protein stability, we asked whether a *UAS-smg-bcd3′UTR* transgene in which the *smg* ORF is fused to the *bcd* 3′ UTR (Figure 4B, top) could restore SMG protein levels in *png* mutants. Because *bcd* mRNA is translated in *png* mutants (Tadros et al., 2003), *UAS-smg-bcd3′UTR* would circumvent a defect in translation of *smg* mRNA. If SMG is translated but unstable in *png* mutants, then the protein produced by *UAS-smg-bcd3′UTR* would also be destabilized. In *png* mutants, the *UAS-smg-bcd3′UTR* transgene produced SMG protein at a level similar to that seen in control embryos (Figure 4B, bottom). Thus, PNG controls the translation of *smg* mRNA but not the stability of SMG protein.

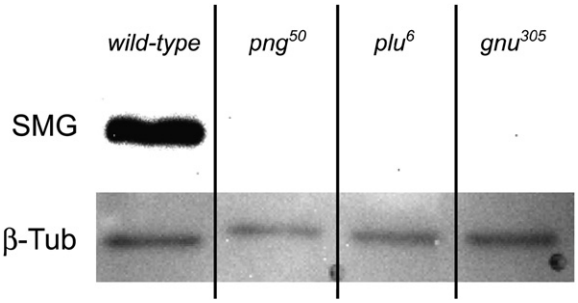

Figure 2. SMG Protein Is Absent in Embryos from *png*, *plu*, and *gnu* Mutant Females

Western blot of extracts from 0- to 3-hour-old embryos probed for SMG and β-tubulin (a loading control).

Translational control is often mediated through the 3′ UTR. To assess whether *smg*'s 3′ UTR is sufficient to confer translational regulation on a heterologous ORF, we constructed two transgenes: *smg5′UTR-GFP-smg3′UTR* (Figure 4C, top) and *UAS-GFP-smg3′UTR* (Figure 4D, top), in which the *smg* ORF was replaced by one encoding green fluorescent protein (GFP). Translation of both transgenic RNAs is activated in wild-type but not in *png* early embryos (Figures 4C and 4D, bottom). Thus, the *smg* 3′ UTR is sufficient to confer *png*-dependent translational regulation on a heterologous ORF. Unexpectedly, in both wild-type and *png* stage 14 oocytes carrying *UAS-GFP-smg3′UTR* there are trace amounts of GFP, whereas *smg5′UTR-GFP-smg3′UTR* is completely silent at this stage. Together these data show that, while there is a weak PNG-independent translational repression element in the *smg* 5′ UTR, the PNG-dependent element resides within the 3′ UTR.

PNG Regulates *smg* mRNA Translation Independently of Its Effects on Cytoplasmic Polyadenylation

We next investigated the role of cytoplasmic polyadenylation in the translational activation of maternal *smg* mRNA. In mature, stage 14 oocytes there were three *smg* mRNA isoforms, each with poly(A) tails approximately 75 nt long (Figure 5A). Following egg activation, these were extended by an additional 100 nt to a total length of ~175 nt in wild-type (Figure 5A). In *png* mutants, the poly(A) tails were extended by only a quarter of the length seen in wild-type (~25 nt; Figure 5A). Only when *smg* transcripts had long poly(A) tails was SMG protein translated (Figure 5B).

To investigate a causal relationship between *smg* mRNA polyadenylation and translation, we overexpressed *Drosophila* poly(A) polymerase (PAP) (Juge et al., 2002) in wild-type and *png* mutants. In wild-type, this caused a significant lengthening of *smg* mRNA poly(A) tails and a 4-fold increase in the levels of SMG protein (Figures 5C and 5D). However, in a *png* mutant background, despite the dramatic increase in *smg* poly(A) tail length, there was no detectable SMG protein (Figures 5C and 5D). As Vardy and Orr-Weaver (2007) have shown that *png* also

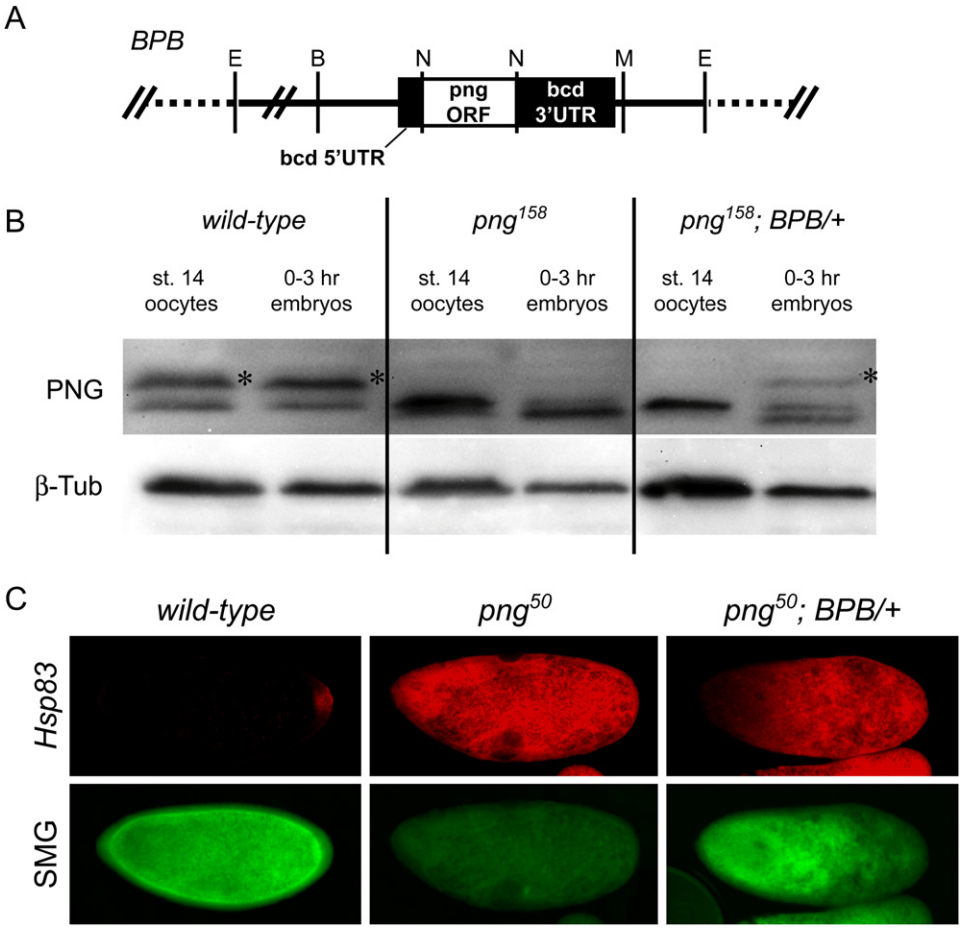

Figure 3. *png* Is Required Following Egg Activation for SMG Expression and Destabilization of Maternal Transcripts

(A) *bcd5′UTR-png-bcd3′UTR* (*BPB*): dashed line represents CaSpeR4 vector sequences; solid line represents genomic DNA flanking the *bcd* 5′ and 3′ UTRs, which are represented by black boxes; white box represents the *png* ORF. E, B, N, and M denote restriction sites for EcoRI, BstEII, NdeI, and MluI, respectively.

(B) Western blot probed for PNG and β-tubulin: PNG protein from *bcd5′UTR-png-bcd3′UTR* is expressed only after egg activation. Full-length PNG is marked with an asterisk (*). Extracts were from stage 14 oocytes and 0- to 3-hour-old embryos of the indicated genotypes.

(C) Whole-mount fluorescent in situ hybridization analysis of *Hsp83* transcripts (top row) and immunostain of SMG protein (bottom row): in *png* embryos, *bcd5′UTR-png-bcd3′UTR* induces mRNA destabilization and SMG expression. Representative 2- to 4-hour-old embryos are shown with the anterior to the left and dorsal toward the top.

promotes translation of *cyclin B* mRNA, we assessed the effects of PAP overexpression on Cyclin B protein levels. As with SMG, levels were increased in wild-type but were not rescued in a *png* mutant (Figure 5D).

As rescue of polyadenylation is not sufficient to rescue translation of *smg* and *cyclin B* mRNA in *png* mutants, PNG must regulate their translation via polyadenylation-independent mechanisms.

Two or More Parallel-Acting Repression Mechanisms Are Likely to Regulate *smg* mRNA Translation

Presence of *smg* mRNA but absence of SMG protein in stage 14 oocytes suggests that, upon egg activation, PNG relieves repression of translation. To identify potential repressors of *smg* mRNA translation, we tested 12 candidate repressors (Figure S2; Table S4): PUMILIO, NANOS, BICOID, BICAUDAL-C, CUP, BRUNO, BRAIN TUMOR, 4E-BP, 4EHP, FMR1, ARMITAGE, and ARGONAUTE-2. Only mutations in *pum* resulted in increased levels of SMG in early embryos (Figure S2). Because *pum* single mutants exhibited increased levels of SMG protein but *pum* mutations were insufficient to suppress the *smg* translational defect of *png* mutants (Figure S2), we speculate that PNG relieves repression by PUM and one or more additional repressors (see Discussion), which act in parallel. We cannot, however, exclude the possibility that PNG prevents the action of an unknown factor that is required, on its own, for repression of *smg* mRNA translation.

SMG Expression Is Not Sufficient to Overcome *png*'s Defect in Maternal Transcript Degradation

To test whether SMG is sufficient to trigger transcript destabilization in *png* mutants, we restored SMG in *png*

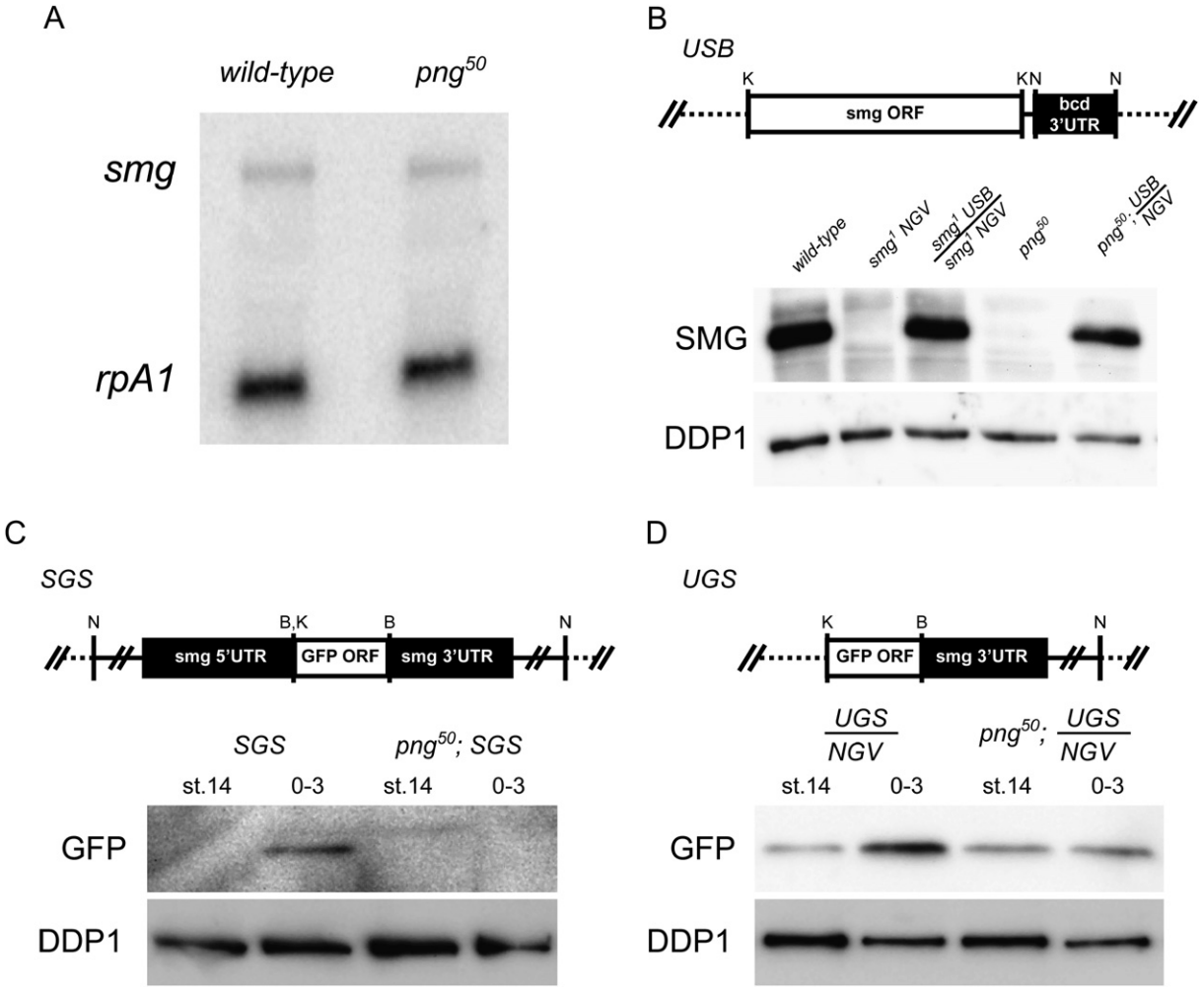

Figure 4. PNG Is Required for the Translation of *smg* mRNA Acting through Its 3′ UTR

(A) Northern blot showing that *smg* mRNA levels are similar in 0- to 3-hour-old wild-type and png^{50} mutant embryos. *rpA1* is a loading control.

(B) *UAS-smg-bcd3′UTR* (*USB*) transgene, which places the *smg* ORF under the translational control of the *bcd* 3′ UTR, is able to restore SMG expression in *png* mutants, as demonstrated in a western blot probed for SMG.

(C and D) *smg5′UTR-GFP-smg3′UTR* (*SGS*) (C) and *UAS-GFP-smg3′UTR* (*UGS*) (D) transgenes are able to reconstitute *png*-dependent translational control as evidenced by western blots probed for GFP. Expression of *UAS-smg-bcd3′UTR* and *UAS-GFP-smg3′UTR* was driven by Nanos-Gal4-VP16 (NGV). In all westerns, extracts were from either 0- to 3-hour-old embryos (0–3) or stage 14 oocytes (st. 14) of the indicated maternal genotype. Blots were also probed for DDP1 (loading control). In all schematics, K, N, and B denote restriction sites for KpnI, NdeI, and BsiWI, respectively; ORFs and UTRs are represented by white and black boxes, respectively; flanking genomic and vector sequences are represented by solid and dashed lines, respectively.

mutants using the *UAS-smg-bcd3′UTR* transgene. We assayed destabilization of *Hsp83*, a SMG-dependent mRNA. Expression of *UAS-smg-bcd3′UTR* mRNA in *smg* mutants rescued maternal *Hsp83* mRNA degradation in the anterior of smg^{1} embryos (Figure S3); thus, *UAS-smg-bcd3′UTR* produces functional SMG protein. However, *Hsp83* mRNA was not destabilized in *png* mutants expressing *UAS-smg-bcd3′UTR* (Figure 6). Thus, PNG is likely to regulate translation or activation of one or more factors that act together with SMG to trigger maternal transcript destabilization (Y in Figure 7).

DISCUSSION

Mechanisms of Maternal mRNA Destabilization

Our gene expression profiling analyses have shown that, in *Drosophila*, a remarkably high fraction (55%) of encoded mRNAs is expressed and loaded into mature oocytes. An earlier estimate of 30% (Arbeitman et al., 2002) was derived from methods biased toward identification of RNAs that are strictly maternally expressed, whereas, in principle, our method identifies all maternally expressed genes, including those also expressed at other

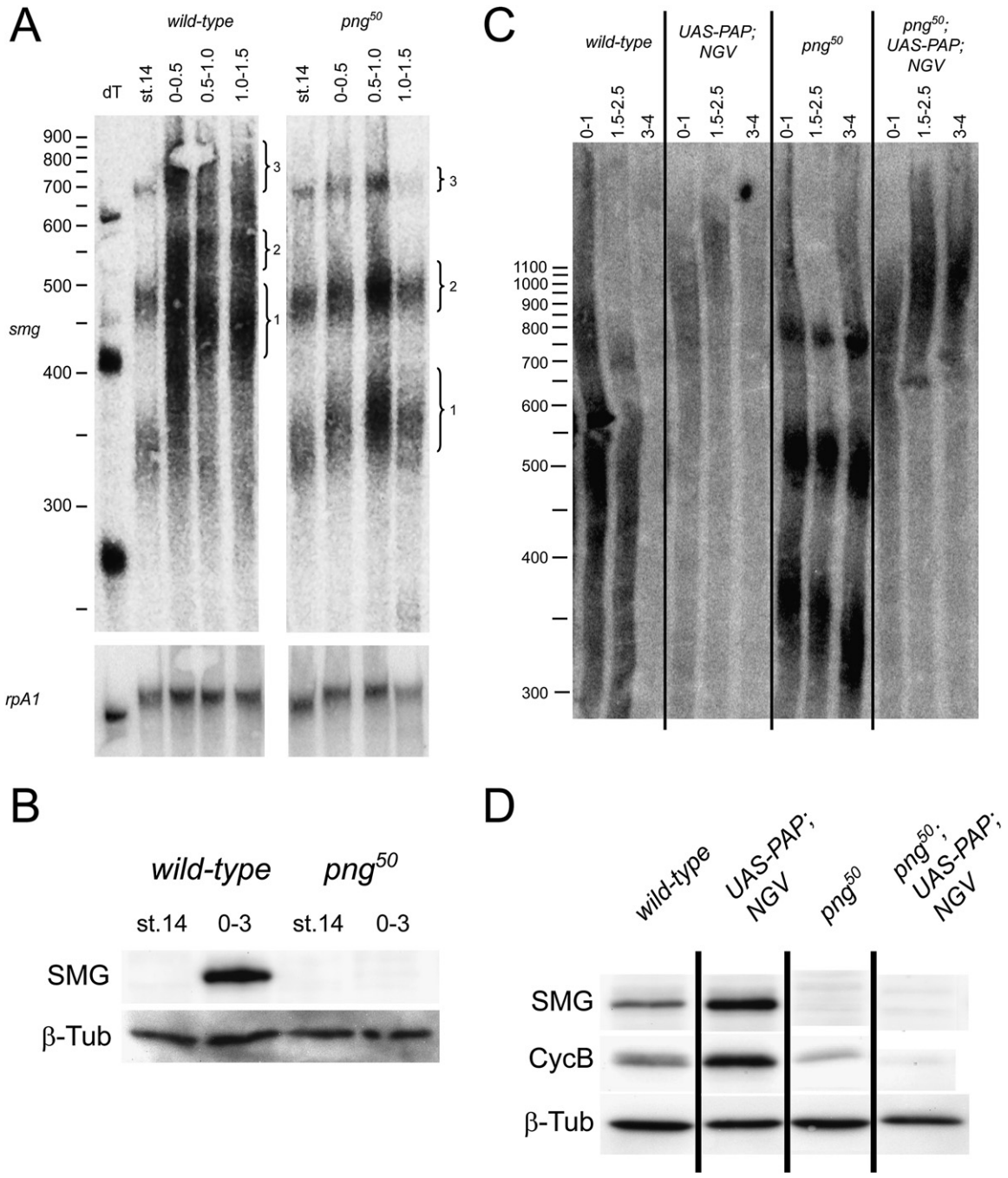

Figure 5. PNG Promotes the Translation of *smg* via Polyadenylation-Independent Mechanisms

(A) Northern blot showing that all three *smg* mRNA isoforms are cytoplasmically polyadenylated following egg activation and that polyadenylation is reduced in *png* mutants. Total RNA from stage 14 oocytes, 0- to 0.5-, 0.5- to 1.0-, and 1.0- to 1.5-hour-old embryos was cleaved with RNase H after hybridization of an antisense oligonucleotide complementary to a site 275 nucleotides 5′ to the first poly(A) addition site. The blot was probed with a *smg* fragment complementary to sequences between the two sites (top panels), stripped, and reprobed for *rpA1* (loading control; bottom panels). The first lane shows total RNA from stage 14 oocytes that had also been hybridized to oligo(dT) prior to RNase H cleavage to reveal the lengths of transcripts lacking poly(A) tails. Brackets denote the size range of each RNA isoform from the 0.5–1.0 hr embryonic time point (labeled 1–3, from smallest to largest). (B) Western blot showing that SMG translation correlates with long poly(A) tails. Extracts were from stage 14 oocytes and 0- to 3-hour-old embryos from wild-type and *png*^{50} mutants. The blot was probed for SMG and β-tubulin. *UAS-PAP* overexpression using the *NGV* driver lengthens *smg* poly(A) tails in both wild-type and *png* mutants (C) but results in increased translation only in the former (D).

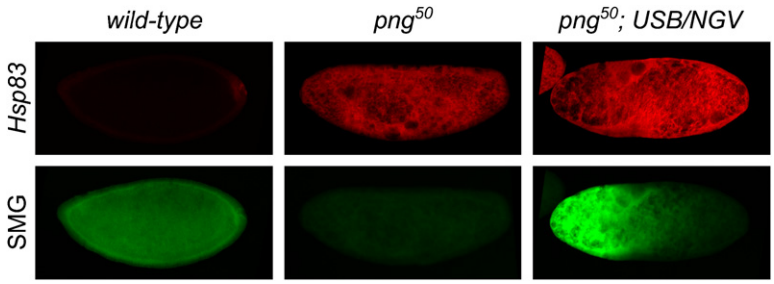

Figure 6. Restoration of SMG Expression in *png* Mutants Is Insufficient to Rescue Destabilization of *Hsp83* mRNA

Whole-mount fluorescent in situ hybridization analysis of *Hsp83* transcripts (top row) and immunostain of SMG protein (bottom row): in *png* embryos, although SMG expression from the *UAS-smg-bcd3′UTR* (*USB*) transgene occurs in the anterior, *Hsp83* mRNA degradation is not induced. Representative embryos of the indicated maternal genotypes from 2- to 4-hour-old collections are shown.

stages or in other cell types. The predicted number of maternal RNAs is likely to increase as more sensitive in situ hybridization methods are used to determine the maternal versus nonmaternal cutoff.

In *Drosophila*, elimination of a subset of maternal transcripts is accomplished through the joint action of two pathways: one is maternally encoded and active in unfertilized eggs; the second requires fertilization and zygotic transcription (Bashirullah et al., 1999; Semotok et al., 2005). Here we have shown that 20% of the maternal mRNAs (more than 1600) are destabilized by the "maternal" pathway. The actual number of maternal transcripts that are destabilized in embryos is, thus, expected to be significantly larger.

Maternal mRNA destabilization in zebrafish depends on *miR-430*, which is absent from the oocytes and is transcribed only after fertilization (Giraldez et al., 2006). *miR-430* therefore functions in a zebrafish pathway equivalent to the *Drosophila* "zygotic" pathway (Bashirullah et al., 1999). Our analyses suggest that, in *Drosophila*, the earlier, "maternal" destabilization pathway does not require miRNAs, as known miRNA binding sites are not enriched in the 3′ UTRs of unstable transcripts. Nonetheless, the fact that several miRNA target sites are enriched in the maternal class as a whole suggests that miRNAs may function in the translational regulation of these transcripts rather than their degradation. It also remains possible that miRNAs participate in the "zygotic" pathway; because this pathway is expected to affect a significantly larger subset of maternal mRNAs than the "maternal" pathway, this would explain the observed enrichment of miRNA targets in the maternal class as a whole.

Quite unexpected was our discovery that SMG is a major regulator of maternal transcript destabilization, being required for elimination of two thirds of the mRNAs that degrade upon egg activation. SMG regulates translation through *cis* elements known as SREs (Dahanukar et al., 1999; Smibert et al., 1999). However, SREs do not mediate SMG-dependent degradation of endogenous transcripts (Semotok et al., 2005). For example, although both *nanos* (SMG-independent) and *Hsp83* (SMG-dependent) mRNAs contain SREs, degradation is SRE dependent only in the case of the former (Semotok et al., 2005; Smibert et al., 1996). Therefore, it is not surprising that SREs are enriched in the unstable class of transcripts but not further enriched in the SMG-dependent subclass.

In summary, though SMG may trigger the degradation of endogenous transcripts through an SRE-independent mechanism, so also SREs may bind a degradation factor other than SMG.

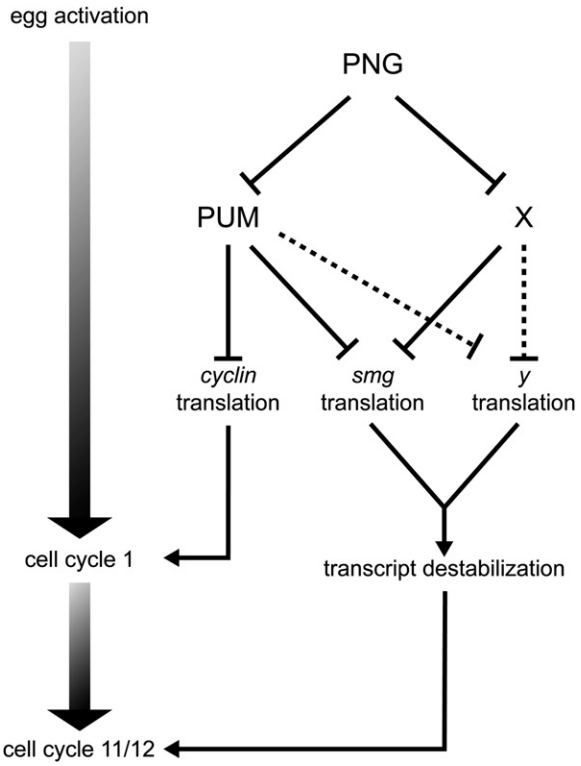

Figure 7. Model for Posttranscriptional Regulation of Maternal Transcripts and Their Role in the Early Embryonic Cell Cycle

PNG is required upon egg activation for the translation of at least three transcripts: *cyclin B*, *smg*, and *y*, which encodes an unknown factor. Though Cyclin translation only requires relief of repression by PUM, *smg* translation requires the relief of at least one additional, parallel-acting repressor (X). The translational activation of the three sets of transcripts is required at different stages of development for distinct purposes. The mitotic Cyclins are necessary for the initial cell cycles. Coincidently, SMG, acting together with Y, triggers the destabilization of maternal transcripts. Transcripts that are targeted by the SMG pathway must be eliminated to allow late cell cycle progression. Solid lines represent steps in the pathway supported by data from this study as well as the published literature; dotted lines represent hypothetical processes.

Role of PNG in mRNA Translation

The discovery that the PNG kinase complex coordinates translation of *smg* mRNA through its 3′ UTR is reminiscent of the role of the Aurora A kinase in translational unmasking of maternal mRNAs during *Xenopus* oocyte maturation (reviewed in Huang and Richter, 2004). In the frog system, Aurora A phosphorylates CPE binding protein (CPEB), which is bound to a 3′ UTR element known as the cytoplasmic polyadenylation element (CPE). CPEB then promotes lengthening of the poly(A) tail, thus facilitating binding of poly(A) binding protein (PABP). PABP in turn binds eIF4G, bringing it into proximity with eIF4E, thus disrupting eIF4E's interaction with the repressor, Maskin. This permits recruitment of the 40S ribosomal complex and initiation of translation. CPEB-mediated regulation of polyadenylation and translation is also crucial later, during early embryogenesis, for cell cycle progression (Groisman et al., 2002).

Though frog Aurora A and fly PNG are both Ser/Thr kinases that function through 3′ UTRs to translationally activate maternal mRNAs, their modes of action differ. Although PNG is required for the polyadenylation of its target transcripts, our data suggest that its role in promoting translation is either "downstream" of or runs "in parallel" to polyadenylation. The distinction between these two mechanisms lies in the interpretation of the fact that lengthening *smg* poly(A) tails results in increased translation in wild-type but not in *png* embryos. A downstream role for PNG could be to transduce a signal linking polyadenylation and translation. For example, in plants, phosphorylation of PABP increases its cooperative binding to poly(A) RNA (Le et al., 2000). Alternatively, PNG might function in a pathway independent of polyadenylation. For example, during *Xenopus* oocyte maturation, Ser/Thr phosphorylation of Maskin is crucial for its dissociation from eIF4E and subsequent translational activation of CPE-bearing transcripts (Barnard et al., 2005). PNG could phosphorylate and cause dissociation of an analogous eIF4E binding protein (there is no clear Maskin ortholog in *Drosophila*).

Vardy and Orr-Weaver (2007) show that PNG promotes translation of Cyclin B. Together with our results on *smg*, this suggests that the previously surmised independent regulation of destabilization and the cell cycle by PNG (Tadros et al., 2003) lies at the level of its targets: *smg* mRNA in the case of destabilization, and *cyclin B* mRNA in that of the early embryonic cell cycle. Though PNG regulates *cyclin B* mRNA translation through the proposed relief of PUM-mediated translational repression, for *smg* mRNA, PNG acts to relieve repression by PUM and one or more proteins that act in parallel (X in Figure 7). Because there are no canonical Nanos response elements (NREs) in the *smg* 3′ UTRs, regulation of *smg* translation by PUM must be indirect or occur via noncanonical NREs. Consistent with either of these possibilities is the recent finding that *smg* mRNA is associated with a transgenic PUM protein fragment in embryonic extracts (Gerber et al., 2006). Also noteworthy is the fact that PUM's repression of one of its known target mRNAs, *hb*, occurs through both polyadenylation-dependent (Wreden et al., 1997) and -independent (Chagnovich and Lehmann, 2001) mechanisms.

We have shown that expression of SMG protein in *png* mutants is not sufficient to restore instability to *Hsp83*, a SMG-dependent maternal mRNA. Thus, destabilization of SMG-dependent maternal mRNAs in eggs from *png* mutant mothers requires one or more additional proteins (Y in Figure 7). PNG may promote the translation of Y, an essential component of the destabilization machinery (as diagramed), or may phosphorylate Y, thus activating the degradation machinery. Global analyses of maternal RNA stability in *png* mutants expressing *UAS-smg-bcd3′UTR* will identify whether any of the PNG-dependent transcripts that are SMG dependent are Y independent.

We note that a third of the unstable maternal mRNAs are SMG independent. PNG function is likely to be required to destabilize a subset of these SMG-independent maternal transcripts. This is suggested by the fact that *nanos* mRNA is fully stabilized in *png* mutants (Tadros et al., 2003) but is only partially stabilized in *smg* mutants (Semotok et al., 2005). Global analyses of maternal RNA stability in *png* mutants will identify all PNG-dependent transcripts.

Biological Significance of Maternal mRNA Destabilization

In *Drosophila* embryos, the transition from maternal to zygotic control of development has been hypothesized to require two processes: elimination of maternal mRNAs and synthesis of zygotic mRNAs. Zygotic transcription is required for cellularization, the hallmark of the *Drosophila* MBT (Merrill et al., 1988; Wieschaus and Sweeton, 1988). However, the functional significance of maternal transcript elimination has remained largely unexplored. *smg* mutants have been shown to fail to progress beyond nuclear cycle 12, never reaching the MBT (Dahanukar et al., 1999), and our computational analyses have shown that the SMG-dependent unstable maternal transcripts are enriched for GO terms related to mitosis and the cell cycle. This enables us to present a model in which elimination of maternal cell cycle mRNAs by SMG is essential for progression through the final syncytial nuclear divisions and, ultimately, the MBT (Figure 7). Detailed cellular and molecular analysis of the *smg* mutant phenotype will be required to test this hypothesis.

SMG homologs exist from yeast to humans, where they function in posttranscriptional regulation (Aviv et al., 2003; Baez and Boccaccio, 2005). Furthermore, the budding yeast homolog Vts1 has been shown to interact with the same *cis* element as SMG (Aviv et al., 2003, 2006). As turnover of maternal mRNAs occurs prior to the MBT in all metazoa, SMG homologs may fulfill a conserved developmental function: targeting a subset of maternal mRNAs for elimination and thus permitting the MBT to occur.

EXPERIMENTAL PROCEDURES

Fly Stocks

Wild-type stocks were *Oregon-R* and w^{1118} (Lindsley and Zimm, 1992). To obtain activated, unfertilized eggs, sterile males of the genotype

T(Y;2)bw $^{DRev\#1}$, *cn bw*$^{DRev\#11}$*mr2/SM6a* (Reed and Orr-Weaver, 1997) were crossed to either w^{1118} or smg^{1}/*Df(Scf)* virgin females (Dahanukar et al., 1999). Other mutants were: png^{50} (Fenger et al., 2000); $png^{12\text{-}158}$, $png^{12\text{-}3318}$ (referred to as png^{158} and png^{3318}, respectively); plu^{6} (Shamanski and Orr-Weaver, 1991); gnu^{305} (Freeman et al., 1986); *pum* alleles *T(1;3)FC8* (*FC8*) and *In(3R)Msc* (*Msc*) (Barker et al., 1992); nos^{BN} (Wang et al., 1994); nos^{L7} (Lehmann and Nüsslein-Volhard, 1991); bcd^{6}, bcd^{12} (Frohnhöfer and Nüsslein-Volhard, 1986); $fmr1^{\Delta 50}$, $fmr1^{\Delta 113}$ (Zhang et al., 2001); $armi^{1}$, $armi^{72.1}$ (Cook et al., 2004); *Df(2L)TE37C-7*; *Bic-C*^{YC33} (Mohler and Wieschaus, 1986); *4E-BP*^{null} (Tettweiler et al., 2005); *4EHP*^{CP53} (Barnard et al., 2005); $cup^{\Delta 212}$ (Nakamura et al., 2004); $aret^{PA}$, $aret^{PD}$ (Schupbach and Wieschaus, 1991); $brat^{fs1}$, $brat^{fs3}$ (Sonoda and Wharton, 2001); and $ago2^{51B}$ (Xu et al., 2004). *nanos-Gal4-VP16* (*NGV*) refers to *P[GAL4::VP16-nos.UTR]* (Van Doren et al., 1998). smg^{1} *NGV* is a recombinant chromosome carrying the smg^{1} mutation and *NGV*. *UAS-PAP* refers to the *UASp-hrg* transgene (Juge et al., 2002).

Gene Expression Profiling

RNA was extracted from staged unfertilized eggs and stage 14 oocytes using a modification of the TRIzol (Invitrogen) method (Neal et al., 2003). Sample quality was evaluated by probing northern blots for known stable (*rpA1*) and unstable (*Hsp83*) transcripts. Total RNA was reverse transcribed as previously described (Neal et al., 2003), except that priming was carried out using random primers rather than oligo-dT in order not to bias the labeling toward mRNAs with long poly(A) tails. The fluorescently labeled cDNA probes were hybridized to 12Kv1 microarray slides obtained from the Canadian *Drosophila* Microarray Centre (http://www.flyarrays.com). These represent 10,500 distinct protein-coding genes, or 77% of the *Drosophila* protein-coding genome. Hybridization and scanning was also as previously described (Neal et al., 2003), using a PerkinElmer/GSI ScanArray 4000 scanner and the ScanArray software. The 16 bit TIFF image files were quantified using QuantArray Version 3 (PerkinElmer) using the adaptive quantification algorithm and analyzed using GeneTraffic Duo 3.2 (Iobion Informatics/Stratagene) after normalization to the known stable transcripts *rpA1* and *rp49*. Quantified microarray data and original TIFF images are available from the Gene Expression Omnibus (GEO) at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/geo/). The 12Kv1 array platform (GEO accession number GPL 1467) has recently been updated with current annotation.

Identification of Maternal mRNAs

Of the 10,500 distinct protein-coding genes on the microarray, 9,257 were analyzed for maternal expression. The averages of all of the raw values for 21 hybridizations of wild-type stage 14 oocyte RNA (three replicates of seven experiments) were sorted from highest to lowest. Maternal expression was assessed at different levels in the list by scanning the Berkeley *Drosophila* Genome Project in situ database (http://www.fruitfly.org/cgi-bin/ex/insitu.pl). RNA from the genes at the top of the table is maternally loaded (90.4% of transcripts with an average raw intensity > 20,000 are maternal; n = 73), whereas RNA from genes at the bottom is absent from early embryos (only 8.2% of those with an average intensity of < 2,000 are maternal; n = 73). *Hsp83* appeared near the top of the list (sixth out of 9,257). Transcripts with average values between 3,500 and 5,000 were mostly maternal (75%; n = 76). As the values decreased, there was a decreasing frequency of maternal transcripts (58.7% between 3,000 and 3,500, n = 172; 46.9% between 2,800 and 3,000, n = 160; 26.2% between 2,500 and 2,800, n = 80; 24.7% between 2,000 and 2,500, n = 81). Using a cutoff value of 3,000, we calculate that 55% of all protein-coding genes are represented in stage 14 oocytes (5,097/9,257 analyzed, extrapolated to 7,745 for the whole genome).

Computational Analysis

5′ UTR, coding, and 3′ UTR sequences were downloaded using the UCSC Table Browser (Karolchik et al., 2004; dm2: April 2004 *Drosophila* genome assembly). SREs were identified by assessing the folding potential of all instances of CNGG and its surrounding sequence using hybrid-ss-min (a newer MFOLD variant; Markham and Zuker, 2005). All occurrences of N_{25}CNGGN_{25} were folded, and instances where the central CNGGN_{1-4} formed the loop region of a hairpin (Aviv et al., 2006) were treated as potential SREs. CNGG conservation was estimated by averaging precomputed Phastcons conservation scores (Siepel et al., 2005) corresponding to the respective CNGG genomic coordinates. Only CNGG regions with mean Phastcons scores greater than 0.8 were considered evolutionarily conserved SREs.

D. melanogaster miRNA and 3′ UTR sequences were downloaded, respectively, from Rfam (Griffiths-Jones, 2004) and UCSC in May 2006. 3′ UTR target sites for all 75 miRNAs were predicted using Targetscan (Lewis et al., 2003). Evolutionary conservation of target sites was assessed using the mean Phastcons score corresponding to the seed region of the UTR. A conservation threshold of 0.8 was selected as the conservation cutoff, as these had the most significant GO (Ashburner et al., 2000) enrichments. The hypergeometric distribution was used to assess overlap between miRNA targets and the various transcript stability classes. Significance of the enrichments was gauged by comparison to randomly generated lists of genes with equal or longer UTRs.

Transcript classes were analyzed for GO terms (Ashburner et al., 2000) using EASE (Hosack et al., 2003). From 43% to 61% of the genes within the distinct classes analyzed had been assigned GO annotations. EASE scores of less than 0.05 were deemed to indicate significant enrichment.

Transgenes

bcd-png-bcd3′UTR was made using an 8.7 kb EcoRI *bcd* genomic rescue fragment (Berleth et al., 1988), which was initially subcloned into the EcoRI site of the Bluescript SK II vector. This made three restriction sites unique: BstEII, which is approximately 1 kb upstream of the *bcd* translation initiation codon; NdeI, which is within the *bcd* ORF; and MluI, which lies 93 bp 3′ of the *bcd* stop codon. The BstEII-NdeI segment was then replaced with PCR fragment 1, while the NdeI-MluI segment was replaced with PCR fragment 2. PCR fragment 1 was generated with a 5′ primer that hybridizes just upstream of the BstEII site and a 3′ primer (that contains an NdeI site in its linker sequence) which hybridizes to a sequence immediately upstream of, and including, the *bcd* start codon. PCR fragment 2 was generated with a 5′ primer which hybridizes immediately downstream of the *bcd* stop codon and a 3′ primer which hybridizes immediately downstream of the MluI site. The resulting plasmid contains an NdeI site in place of the *bcd* ORF. The *png* ORF (second codon to stop codon) was amplified from genomic DNA with primers containing the NdeI site in their linker sequences and was inserted into the NdeI site in the above plasmid. The resulting EcoRI fragment was then cloned into the CaSpeR4 vector for germline transformation.

UAS-smg-bcd3′UTR was made by amplifying the *smg* ORF with KpnI-containing primers from cDNA and cloning it into the KpnI site of pUASp (Rørth, 1998). The *bcd* 3′ UTR was inserted into the downstream NotI site using the same primers as in Zhang et al. (2004), except with NotI linkers.

smg5′UTR-GFP-smg3′UTR was made from a *smg* genomic rescue construct (Dahanukar et al., 1999) in which the *smg* ORF was replaced with a BsiWI restriction site using a similar strategy to *bcd5′UTR-png-bcd3′UTR*. This site was then used to insert the GFP ORF (Zacharias et al., 2002), which was amplified using a 5′ primer with both BsiWI and KpnI linkers, and a 3′ primer with BsiWI alone. This was then cloned into the CaSpeR4 vector for germline transformation.

UAS-GFP-smg3′UTR was made by subcloning the KpnI-NotI fragment from the above *smg5′UTR-GFP-smg3′UTR* construct into pUASp. This fragment contains the GFP ORF followed by 2 kb of genomic DNA downstream of the *smg* stop codon. This allows for the production of transcripts from all three of *smg*'s polyadenylation sites.

Protein Methods

Extract from approximately 20 embryos/ooctyes was loaded per lane. Primary antibodies were: guinea pig anti-PNG (1:2,000; Fenger et al.,

2000); mouse anti-β-tubulin E7 (1:500; Developmental Studies Hybridoma Bank, Iowa City); guinea pig anti-DDP1 (1:5,000; M. Nelson, H. Luo, C.A.S., and H.D.L., unpublished data); guinea pig anti-SMG (1:10,000; Cedarlane Laboratories, raised against the same antigen used in Smibert et al., 1999); rabbit anti-GFP ab290 (1:2,000; Abcam); and mouse anti-Cyclin B F2F4 (1:5; Developmental Studies Hybridoma Bank). Secondary goat anti-guinea pig horseradish peroxidase (HRP), goat anti-mouse HRP, and goat anti-rabbit HRP were used at a 1:5,000 dilution (Jackson). Signal was visualized with FluorChem using the ECL detection system (Pierce) and associated software (Alpha Innotech). Whole-mount immunostaining of embryos used guinea pig anti-SMG antibody at a dilution of 1:500.

RNA Methods

Embryos for whole-mount in situ hybridization were fixed and hybridized with DIG-labeled *Hsp83* antisense RNA probes, which were detected using either HRP-based tyramide signal amplification (http://www.utoronto.ca/krause) or conventional alkaline phosphatase-based (Bashirullah et al., 1999; Ding et al., 1993) methods. Northern blot analyses and RNase H cleavage assays were performed as described previously (Semotok et al., 2005).

Supplemental Data

Supplemental Data include three figures and four tables and can be found with this article online at http://www.developmentalcell.com/cgi/content/full/12/1/143/DC1/.

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