

Drosophila TIF-IA is required for ribosome synthesis and cell growth and is regulated by the TOR pathway

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Synthesis of ribosomal RNA (rRNA) is a key step in ribosome biogenesis and is essential for cell growth. Few studies, however, have investigated rRNA synthesis regulation in vivo in multicellular organisms. Here, we present a genetic analysis of transcription initiation factor IA (TIF-IA), a conserved RNA polymerase I transcription factor. *Drosophila melanogaster* *Tif-IA*^{-/-} mutants have reduced levels of rRNA synthesis and sustain a developmental arrest caused by a block in cellular growth. We find that the target of rapamycin (TOR) pathway regulates TIF-IA recruitment to rDNA. Further-

more, we show that the TOR pathway regulates rRNA synthesis in vivo and that TIF-IA overexpression can maintain rRNA transcription when TOR activity is reduced in developing larvae. We propose that TIF-IA acts in vivo as a downstream growth-regulatory target of the TOR pathway. Overexpression of TIF-IA also elevates levels of both 5S RNA and messenger RNAs encoding ribosomal proteins. Stimulation of rRNA synthesis by TIF-IA may therefore provide a feed-forward mechanism to coregulate the levels of other ribosome components.

Introduction

Ribosomal RNA (rRNA) transcription is a key step in the synthesis of ribosomes and occurs through the control of RNA polymerase I (Pol I; Grummt, 2003; Moss, 2004; Russell and Zomerdijk, 2005). Over the last several decades, studies in unicellular systems, particularly *Escherichia coli* and *Sacharomyces cerevisiae*, have examined the mechanisms by which rRNA synthesis is regulated (Nomura, 1999; Paul et al., 2004). More recent studies in mammalian cell culture have also identified mechanisms via which Pol I is controlled. However, few studies have addressed how rRNA synthesis is regulated in vivo during the growth of a multicellular animal. Given that ribosome biogenesis links extracellular signals to the control of cell growth, identifying the mechanisms that operate in vivo should provide key insights into the control of cell and tissue growth.

Nutrient availability is a key determinant of cell and organismal growth. In eukaryotes, the target of rapamycin (TOR) kinase pathway is a major growth-regulatory pathway activated in response to nutrient availability (Wullschleger et al., 2006). Although biochemical and genetic analyses have defined the signaling inputs to TOR, the outputs via which TOR drives growth are not fully understood. An extensive literature suggests that TOR controls growth by stimulating mRNA translation, particularly through the effectors ribosomal protein (RP), S6 kinase (S6K), and translation initiation factor *4E-binding protein*. But these targets are unlikely to explain all the effects of TOR in vivo. For example, *Drosophila melanogaster* TOR mutants are lethal and have marked growth defects, whereas *S6K* and *4E-binding protein* mutants are viable and have mild growth phenotypes (Montagne et al., 1999; Oldham et al., 2000; Zhang et al., 2000; Miron et al., 2001). Hence, other downstream targets and metabolic processes must additionally contribute to the effects of TOR in vivo.

Studies in yeast and mammalian cell culture indicate that regulation of rRNA synthesis is a conserved TOR function (Zaragoza et al., 1998; Powers and Walter, 1999; Hannan et al., 2003; Tsang et al., 2003; Claypool et al., 2004; James and Zomerdijk, 2004; Mayer et al., 2004; Li et al., 2006). A few studies have described mechanisms by which TOR can affect

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Abbreviations used in this paper: eIF4E, eukaryotic translation initiation factor 4E; ETS, external transcribed spacer; GPDH, glycerol 3-phosphate dehydrogenase; RP, ribosomal protein; rRNA, ribosomal RNA; S6K, S6 kinase; TIF-IA, transcription initiation factor IA; TOR, target of rapamycin; UAS, upstream activator sequence; UBF, upstream binding factor.

The online version of this article contains supplemental material.

Pol I activity; however, these have yielded conflicting results. Studies in yeast and mammalian cell culture reported that TOR regulated the ability of a conserved transcription factor, transcription initiation factor IA (TIF-IA; or Rrn3p, the yeast homologue of TIF-IA), to recruit Pol I to rDNA (Claypool et al., 2004; Mayer et al., 2004). But another paper on mammalian cells found that TIF-IA is dispensable for TOR-dependent regulation of rRNA synthesis and suggested that a different Pol I factor, upstream binding factor (UBF), was a target of TOR signaling (Hannan et al., 2003). Finally, a recent paper showed that TOR associates with the rDNA in yeast, suggesting that regulation of Pol I by TOR is direct (Li et al., 2006). TOR may therefore control rRNA synthesis through multiple mechanisms. Whether or not TOR regulates rRNA synthesis in vivo in animals and what mechanisms may operate in this context have not been examined.

Here, we examine the role of the *D. melanogaster* homologue of the conserved Pol I factor TIF-IA in the control of ribosome synthesis and growth. We show that TIF-IA is required for rRNA synthesis and cell and organismal growth and that TIF-IA functions downstream of the TOR pathway in vivo. We also provide evidence that stimulation of rRNA synthesis by TIF-IA can control the levels of other ribosome components.

Results and discussion

Tif-IA is required for cell and organismal growth

A recessive lethal *P*-element line, KG06857, was available from a public stock center. This line contains a *P*-element insertion in the 5' region of the TIF-IA gene, which reduced *TIF-IA* mRNA expression in homozygote L1 larvae compared with the wild type (Fig. 1 A, left). *Tif-IA*^{KG06857} homozygote (*Tif-IA*^{-/-}) larvae had low levels of pre-rRNA synthesis (Fig. 1 A, right). Phenotypically, *Tif-IA*^{-/-} mutant animals developed through embryogenesis and hatched at the normal time. But as larvae, they failed to develop and exhibited a growth arrest phenotype, surviving for up to 8 d as arrested L1 larvae (Fig. 1 B). This growth arrest phenotype was fully reversed by ubiquitous expression of a TIF-IA cDNA transgene in mutant larvae using the GAL4–upstream activator sequence (UAS) system (Fig. S1 A, available at <http://www.jcb.org/cgi/content/full/jcb.200709044/DC1>). Using the hsFlp–GAL4 method, mosaic expression of TIF-IA cDNA in the larval fat body of *Tif-IA*^{-/-} larvae led to a cell-autonomous rescue of growth (Fig. 1 D). Conversely, mosaic expression of a TIF-IA RNAi construct in wild-type larva cells autonomously inhibited growth in the fat body (Fig. 1 E). Thus, the developmental arrest we observed in the *Tif-IA*^{-/-} mutants was caused by a cell-autonomous defect in growth. We also found that RNAi-mediated knockdown of TIF-IA in cultured *D. melanogaster* S2 cells reduced cell size and caused cells to accumulate in G1 phase of the cell cycle (Fig. S1 B). Thus, TIF-IA activity is necessary for proper rRNA synthesis and cell growth during development.

A recent study reported that loss of TIF-IA in mouse cells induced p53-dependent cell cycle arrest and cell death (Yuan et al., 2005). These findings are consistent with the emerging view that nucleolar disruption triggers a p53-dependent checkpoint in mammalian cells (Horn and Vousden, 2004). We stained

larval tissue from *D. melanogaster Tif-IA* mutants with an anti-cleaved caspase 3 antibody and found no signs of apoptosis (unpublished data). Moreover, the growth arrest phenotypes observed in *TIF-IA*^{-/-} larvae were not suppressed by genetic deletion of the *D. melanogaster* homologue of p53 (Fig. 1 C). This is in contrast to mammalian cells, where the apoptosis and cell cycle arrest induced by loss of TIF-IA was reversed by loss of p53 (Yuan et al., 2005). These studies suggest that the link between nucleolar viability and p53 function may not be present in *D. melanogaster*. This may be because Mdm2 and Arf, the factors thought to link disruption of the nucleolus to p53 activation in mammalian cells, have no obvious homologues in *D. melanogaster*.

Overexpression of TIF-IA is sufficient to increase pre-rRNA synthesis

Ubiquitous overexpression of TIF-IA in whole larvae, using a *da-GAL4* driver, increased levels of pre-rRNA as measured by quantitative RT-PCR (Fig. 1 F). Similarly, expression of TIF-IA in the posterior compartment of the wing imaginal disc also increased pre-rRNA levels as measured by in situ hybridization (Fig. 1 G). We used L-[methyl³H]methionine to carry out pulse-chase labeling of RNA and observed normal rates of rRNA processing in TIF-IA–overexpressing larvae (Fig. S2, available at <http://www.jcb.org/cgi/content/full/jcb.200709044/DC1>). Hence, the increases in pre-rRNA levels we observed were caused by a specific effect on rRNA transcription and did not simply reflect a block in rRNA processing. Thus, TIF-IA overexpression alone is sufficient to increase Pol I transcriptional activity in vivo.

Nutrition and the TOR pathway regulate rRNA synthesis in vivo

We next examined the control of TIF-IA function, focusing on the TOR pathway as a potential regulatory input. TOR activity can be suppressed in larvae by starvation for dietary protein (Oldham et al., 2000). We found that protein starvation induced a marked decline in levels of both total rRNA (Fig. 2 A) and pre-rRNA synthesis (Fig. 2, B and C). We also saw a similar reduction in rRNA synthesis in both homozygous TOR mutant larvae (Fig. 2 D) and larvae that had been fed the TOR inhibitor rapamycin for 24 h (not depicted). In contrast, Gal4-mediated overexpression of the small G protein Rheb, an upstream activator of TOR, was sufficient to increase pre-rRNA synthesis in whole larvae (Fig. 2 E). Similarly, *en-Gal4*–driven expression of Rheb in the posterior compartments of larval wing imaginal discs also increased rRNA synthesis (Fig. 2 F). Finally, Rheb overexpression increased nucleolar size, which is an index of ribosome biogenesis, as measured by staining with fibrillarin, a nucleolar protein (Fig. 2 G). Thus, the TOR pathway is both necessary and sufficient for regulating rRNA synthesis in developing *D. melanogaster* tissues.

TIF-IA functions downstream of the TOR pathway

We examined whether TIF-IA might function as a downstream target of the TOR pathway. TIF-IA acts by binding Pol I and recruiting it to the rDNA promoter (Bodem et al., 2000; Moorefield

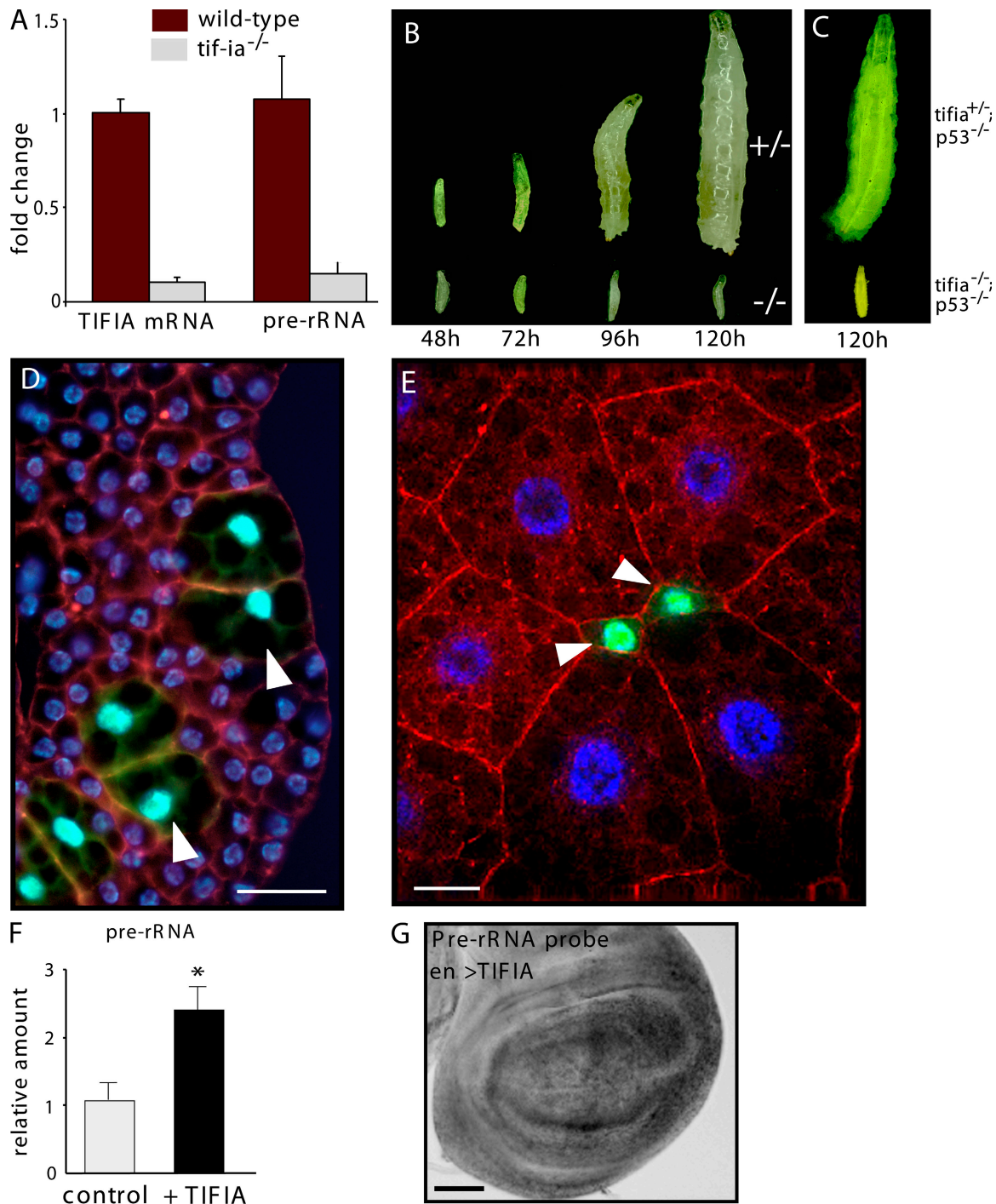


Figure 1. TIF-IA is required for cell and organismal growth. (A) Levels of TIF-IA mRNA and pre-rRNA were measured by quantitative RT-PCR, using RNA isolated from either wild-type or *Tif-IA*^{-/-} mutant larvae. Data were corrected for levels of GPDH mRNA. Data are mean (\pm SEM) fold changes compared with wild type ($n = 6$). (B) *Tif-IA*^{-/-} mutant larvae are growth arrested. Images of *Tif-IA* heterozygote (+/-) and *Tif-IA* homozygous mutant larvae (-/-) at different stages (48–120 h) of larval development are shown. (C) Loss of p53 has no effect on the growth arrest phenotype seen in TIF-IA mutant larvae. Images of *Tif-IA*^{+/-}; *p53*^{-/-} (top) or *Tif-IA*^{-/-}; *p53*^{-/-} (bottom) larvae at 120 h of development are shown. (D) The hsFlp-GAL4 system was used to generate mosaic expression of GFP-marked cells overexpressing TIF-IA (arrowheads) in the larval fat body of *Tif-IA*^{-/-} mutant animals (red, phalloidin; blue, DAPI). Bar, 25 μ m. (E) The hsFlp-GAL4 system was used to generate mosaic expression of both GFP and a TIF-IA RNAi construct in the polypliod cells of the larval fat body (arrowheads; green, GFP; red, phalloidin; blue, DAPI). Bar, 25 μ m. (F) quantitative RT-PCR was used to measure levels of pre-rRNA in either control larvae or larvae overexpressing UAS-TIF-IA under the control of the *da*-GAL4 driver. Data were corrected for levels of GPDH mRNA. Data are mean \pm SEM. *, $P < 0.05$ versus control ($n = 7$ –8). (G) An *en*-GAL4 driver was used to express a TIF-IA cDNA in the posterior compartment of the developing larval wing imaginal disc. Levels of pre-rRNA were then measured in wandering L3 wing discs by in situ hybridization using a probe to the ETS region of the pre-rRNA precursor. Posterior is to the right. Bar, 50 μ m.

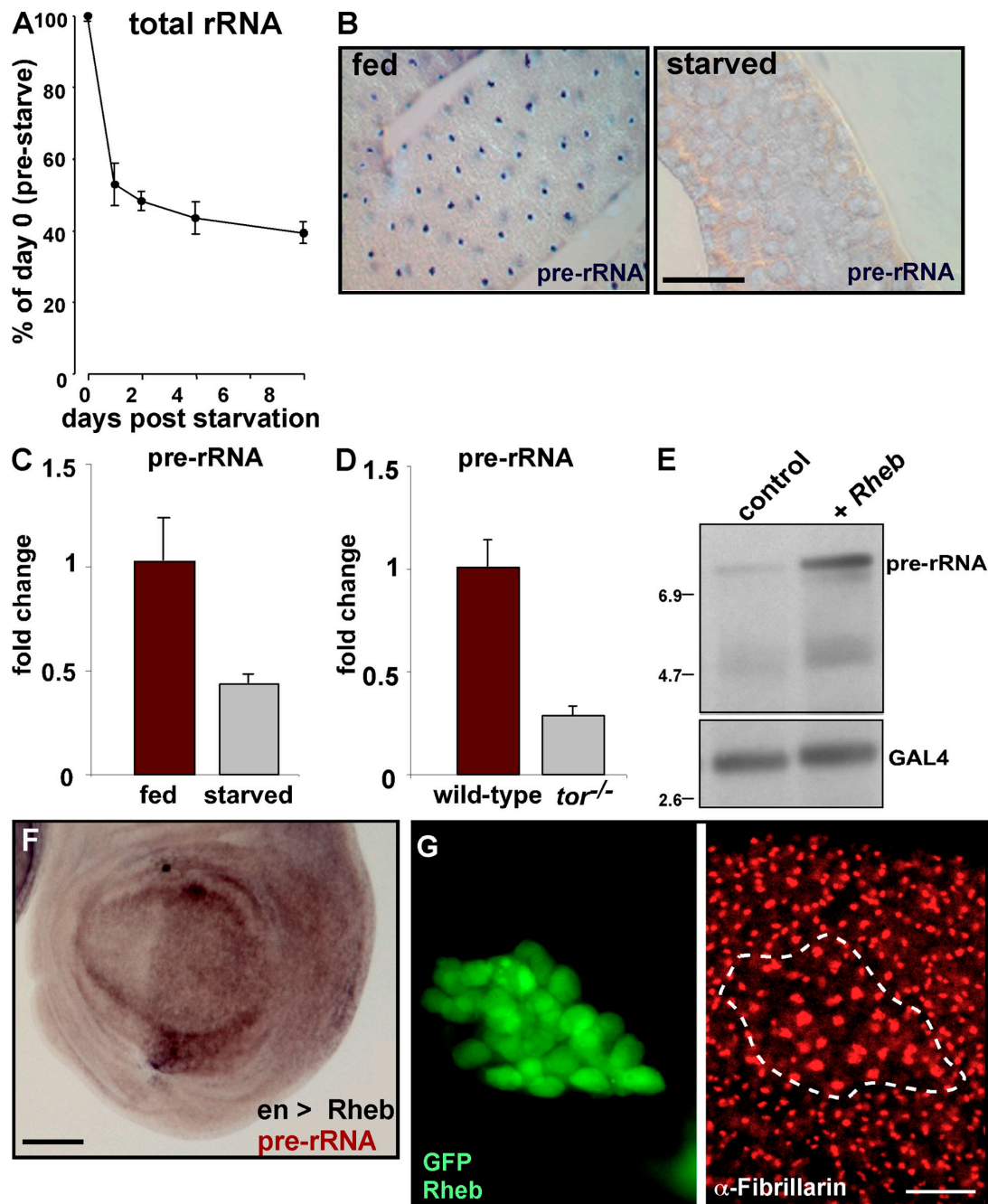


Figure 2. **The *D. melanogaster* nutrient-TOR pathway regulates rRNA synthesis and ribosome biogenesis.** (A) Wild-type larvae were starved and, at the indicated times, total RNA was isolated from equal numbers of larvae per time point, and then levels of rRNA were quantitated from ethidium bromide-stained agarose/formaldehyde gels. Data are mean (\pm SEM) percentage changes in rRNA levels relative to nonstarved animals (day 0 time point; $n = 3-6$ per time point). (B) Levels of pre-rRNA were measured in fed or 4-d starved larvae by in situ hybridization. A representative image of the gut is shown for both samples. Bar, 25 μ m. (C and D) Levels of pre-rRNA were measured by quantitative RT-PCR using RNA isolated from either wild-type or starved larvae (C) or wild-type or *tor^{-/-}* mutant larvae (D). Data are mean (\pm SEM) fold changes versus fed larvae ($n = 3$). Data were corrected for levels of dMyc mRNA. (E) The hsFlp-GAL4 system was used to overexpress Rheb transgene throughout developing larvae. Levels of pre-rRNA and GAL4 (as loading control) were measured by Northern blot. Reference DNA fragment sizes are indicated (kb). (F) An *en-GAL4* driver was used to express a Rheb transgene in the posterior compartment of wing imaginal discs. Levels of pre-rRNA were then measured by in situ hybridization. Posterior is to the right. Bar, 50 μ m. (G) The hsFlp-Gal4 system was used to generate cell clones coexpressing GFP and Rheb in developing wing imaginal discs. Discs were stained with an antibody to fibrillaritin (red). The dashed line shows the clone outline. Bar, 20 μ m.

et al., 2000; Miller et al., 2001; Claypool et al., 2004; Mayer et al., 2004). Using the DamID technique, we examined whether TIF-IA localization to rDNA might be TOR regulated (van Steensel and Henikoff, 2000; Grewal et al., 2005). A bacterial

DNA methylase fused to *TIF-IA* was used to locally mark TIF-IA-associated genomic loci after transfection and low-level expression in *D. melanogaster* Kc cells. After isolation of genomic DNA and DpnII digestion, we used quantitative PCR

to analyze the levels of methylated fragments. We found that levels of methylation were significantly higher in Dam-TIF-IA-transfected cells than in cells transfected with Dam alone (Fig. 3 A). This is consistent with TIF-IA associating with rDNA. This signal in Dam-TIF-IA-transfected cells was attenuated when cells were treated with rapamycin, an inhibitor of the TOR kinase (Fig. 3 A). These data indicate that TOR controls the ability of TIF-IA to associate with the rDNA locus in *D. melanogaster* cells.

We next examined the relationship between TOR and TIF-IA function in animals. As described earlier, TIF-IA overexpression was sufficient to increase Pol I activity and elevate pre-rRNA levels in larvae. Interestingly, we also found that TIF-IA overexpression increased pre-rRNA levels in both protein-starved and rapamycin-treated larvae to the same degree as in control larvae (Fig. 3, B and C). Thus, TIF-IA overexpression can prevent the inhibition of pre-rRNA synthesis that normally results from reduced TOR activity. These data suggest that TIF-IA functions downstream of TOR in the regulation of rRNA synthesis.

Our findings point to *D. melanogaster* TIF-IA as a growth-regulatory target of the TOR pathway in vivo. Based on previous in vitro data in mammalian cells and yeast (Grummt, 2003; Moss, 2004), this role of TIF-IA seems to be conserved. Whether the mechanisms via which TOR regulates TIF-IA are also conserved is unclear, although they may involve phosphorylation of either TIF-IA or Pol I (Fath et al., 2001; Cavanaugh et al., 2002; Mayer et al., 2004). In mammals, another Pol I-associated factor, UBF, is also regulated by growth factors (Grummt, 2003; Moss, 2004). However, *D. melanogaster* contains no obvious homologue to UBF, and hence the potential regulation of rRNA synthesis by TOR through such a factor is unclear in flies.

Overexpressed TIF-IA controls the levels of RP mRNA

A recent study in yeast showed that constitutive Pol I activity could maintain rRNA transcription and synthesis of all RP mRNAs and 5S RNA levels under situations that normally inhibit ribosome synthesis, such as nutrient deprivation (Laferte et al., 2006). We therefore examined whether increased TIF-IA activity was sufficient to regulate other aspects of ribosome synthesis in developing larvae. We measured levels of mRNAs encoding 27 different RPs and found that 21 were significantly increased by TIF-IA overexpression (B). We also found that TIF-IA overexpression increased levels of 5S RNA, a ribosomal component that is normally transcribed by RNA Pol III (Fig. 4 A). Finally, we examined a selection of other genes encoding Pol I and III components and rRNA processing factors. Of these transcripts, only levels of Brf, an RNA Pol III factor, and two rRNA processing genes, fibrillarin and NNP-1, were significantly increased by TIF-IA overexpression (Fig. 4 C). These data suggest the interesting possibility that elevation of TIF-IA expression not only increases Pol I activity but can also drive a feed-forward mechanism that couples the synthesis of 45S rRNA with increases in 5S RNA and RP mRNA levels.

Thus, both *D. melanogaster* and yeast appear able to co-regulate the levels of mRNAs encoding RPs through the stim-

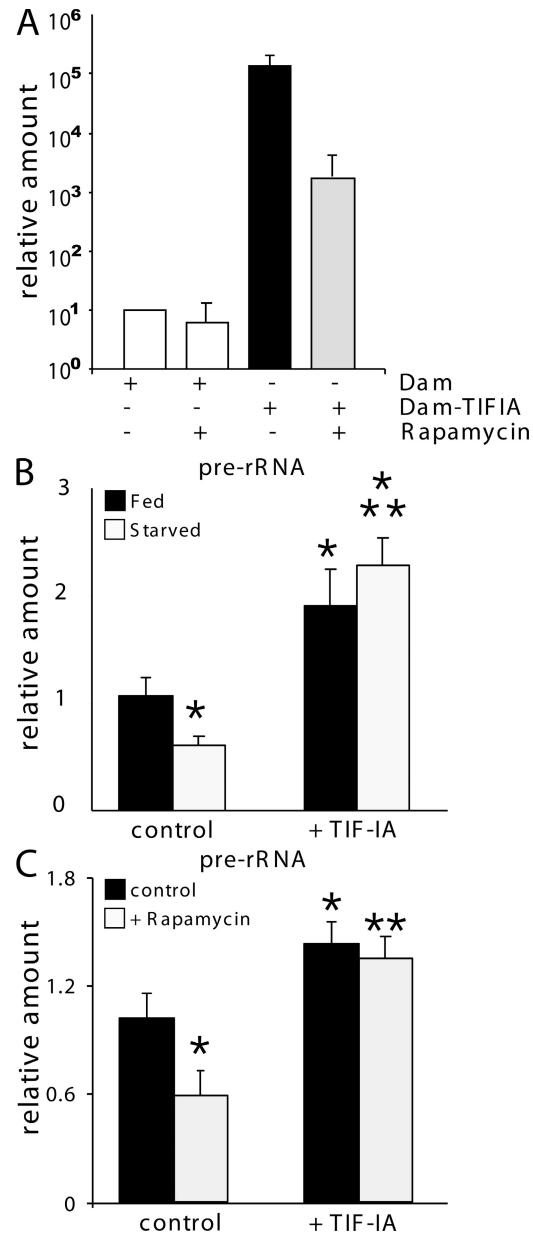


Figure 3. TIF-IA functions downstream of the TOR pathway. (A) The localization of TIF-IA to rDNA was measured using the DamID technique. *D. melanogaster* Kc cells were transfected with either Dam alone or a Dam-TIF-IA fusion. Cells were then treated with DMSO (control) or rapamycin for 16 h as indicated. Genomic DNA was isolated and digested with DpnII and identical amounts per sample were analyzed by quantitative PCR. Data represent mean (\pm SEM) fold changes (log scale) compared with Dam alone, the control cells ($n = 3$). (B and C) Quantitative RT-PCR was used to measure levels of pre-rRNA in either control larvae or larvae overexpressing UAS-TIF-IA under the control of the *da-GAL4* driver. In B, larvae were either fed for 2 d or starved for an additional day. In C, larvae were fed for 2 d and then transferred to new vials for 24 h with either normal food containing 0.1% DMSO control or food supplemented with 10 μ M rapamycin. Data are mean (\pm SEM) fold changes in pre-rRNA levels versus fed controls ($n = 4$). (B) *, $P < 0.05$ versus fed control; **, $P < 0.05$ versus starved control. (C) *, $P < 0.05$ versus DMSO control; **, $P < 0.05$ versus rapamycin control.

ulation of Pol I. How might this happen? One hypothesis is that cells can sense either the absolute levels of rRNA or the process of rRNA synthesis to trigger changes in the amounts

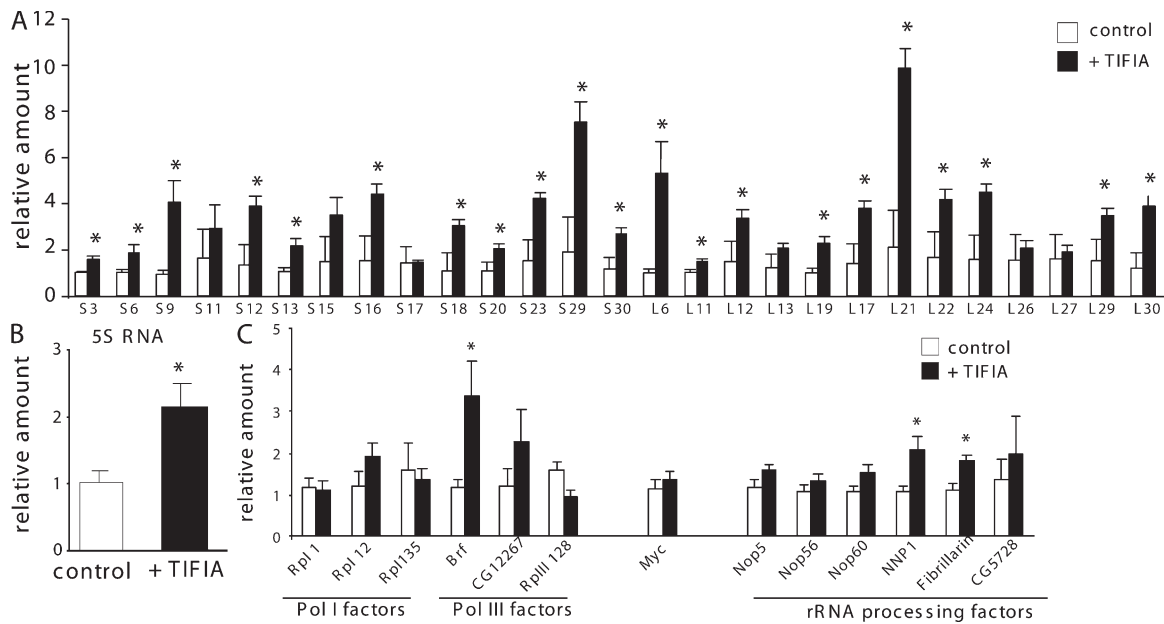


Figure 4. *D. melanogaster* TIF-IA regulates rRNA synthesis and controls the levels of other components of the ribosome. (A–C) A *da-GAL4* driver was used to express TIF-IA ubiquitously in developing larvae. Total RNA was extracted from control and TIF-IA-overexpressing larvae. Quantitative RT-PCR was used to measure levels of indicated transcripts. Data are presented as fold changes compared with wild type and represent the mean \pm SEM ($n = 7$ –8). Data were corrected for levels of GPDH mRNA. *, $P < 0.05$ versus control.

of other ribosome components. Given that dozens of proteins and small RNAs are required to synthesize and process rRNA, changes in the activity, levels, or nuclear localization of any of these could be involved in the control of RP mRNA and 5S RNA levels. In addition, many noncoding RNAs, as well as the mature rRNAs themselves, are produced as a result of transcription at the rDNA locus. Thus an intriguing possibility is that these RNAs may contribute to the feed-forward regulation.

Overexpressed TIF-IA is not sufficient to drive protein synthesis or cell growth

We also examined whether TIF-IA overexpression could increase levels of ribosome subunits, protein synthesis, and cell growth in developing larvae. We measured levels of 40S and 60S ribosome subunits in larval extracts using sucrose gradient fractionation. These experiments showed no marked differences in subunit levels between control and TIF-IA-overexpressing larvae (Fig. 5 A). Similarly, we found no significant change in protein synthesis caused by TIF-IA overexpression (Fig. 5 B). Under the same conditions, Rheb overexpression, which activates TOR and stimulates rRNA synthesis, induced a 50–80% increase in protein translation (Hall et al., 2007). We finally examined whether TIF-IA overexpression was sufficient to alter cell growth or cell cycle progression in developing larvae. We used the *hsFlp*–*Gal4* system to generate random cell clones that overexpressed TIF-IA in developing wing imaginal discs. Using flow cytometry, we found no difference in either cell size or cell cycle phasing in TIF-IA-overexpressing cells (Fig. 5 C). Moreover, we did not see any increase in clone areas between control and TIF-IA-overexpressing clones (Fig. 5 D). In similar assays, the activation of TOR by over-

expression of Rheb increased clone sizes by $\sim 60\%$ (Saucedo et al., 2003).

Therefore, despite stimulating pre-rRNA synthesis and increasing the levels of RP mRNA, TIF-IA overexpression was not sufficient to drive ribosome or protein synthesis or to increase cell growth. These findings in *D. melanogaster* differ from those in yeast, where the stimulatory effects of constitutive Pol I activity on levels of 5S RNA and RP mRNA did maintain 40S and 60S ribosome subunit levels after inhibition of TOR (Laferte et al., 2006). Thus, other, possibly TOR-regulated, events limit production of active ribosomes in *D. melanogaster*. One possibility is that the translational control of RP mRNAs is limiting. In contrast to yeast, *D. melanogaster* and other higher eukaryotic RP mRNAs contain a structural motif, the 5'-terminal oligopyrimidine tract, which controls their translation and allows the coordinated synthesis of RPs, particularly in response to TOR signaling (Meyuhas and Hornstein, 2000). Other TOR-regulated steps, such as the maturation and nuclear export of ribosomes (Honma et al., 2006; Pelletier et al., 2007), may also be limiting.

One intriguing possibility is that expression of a defined set of limiting downstream TOR targets may be sufficient to mediate the strong induction of protein synthesis induced by activating TOR in *D. melanogaster*. We addressed this by examining the effects of coexpressing either S6K or the eukaryotic translation initiation factor 4E (eIF4E) with TIF-IA. Increases in both S6K and eIF4E activity have been suggested as key downstream effectors of the TOR pathway in the control of cell growth (Wullschlegel et al., 2006); however, we did not see any marked changes in protein synthesis (Fig. 5 E). Further studies will be required to identify the TOR effectors that are required to cooperate with TIF-IA to drive ribosome synthesis and mRNA translation. Nevertheless, given our observations in *D. melanogaster* and previous work in

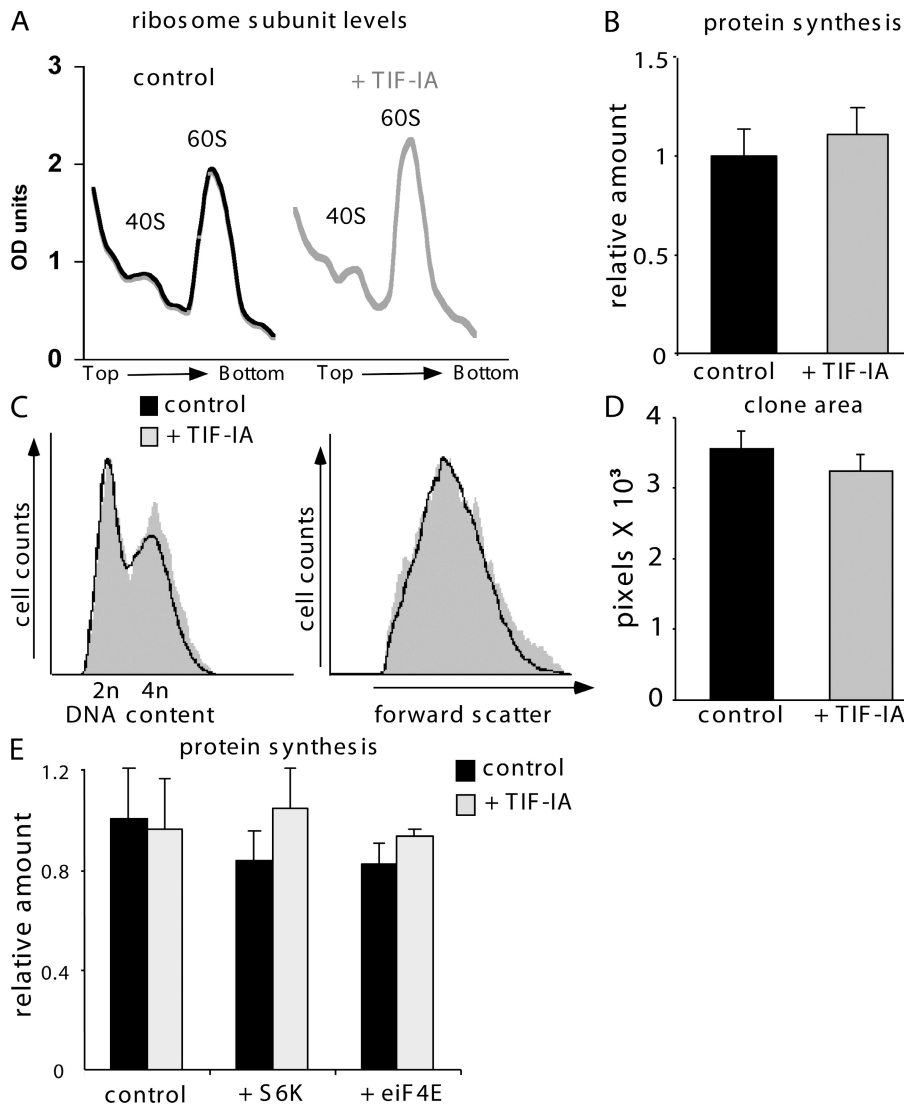


Figure 5. Overexpression of TIF-IA is not sufficient to drive ribosome synthesis, mRNA translation, or cell growth. (A) 40S and 60S ribosome particles were separated by sucrose gradient centrifugation and their levels determined by measuring absorbance at 260 nm. (B) Using a *da-GAL4* driver, levels of protein synthesis were measured in either control larvae or larvae in which TIF-IA was ubiquitously overexpressed (+ TIF-IA). Data represent the mean (\pm SEM) percentage change in radiolabeled amino acid incorporation compared with control larvae. (C) Flow cytometry profiles of wing imaginal disc cells. Cell cycle phasing (left) or cell size (right) comparisons of GFP-marked TIF-IA-overexpressing cells (gray traces) with surrounding nonmarked wild-type cells (black traces) are shown. (D) Data represent the mean (\pm SEM) percentage changes in clone area in either control or TIF-IA-overexpressing cell clones in the wing imaginal disc ($n = 200$). (E) Using a *da-GAL4* driver, levels of protein synthesis were measured in control larvae and larvae in which a UAS-TIF-IA transgene was ubiquitously overexpressed (+ TIF-IA). In addition, either UAS-S6K (+S6K) or UAS-eIF4E (+eIF4E) transgenes were co-expressed. Data represent the mean (\pm SEM) percentage changes in radiolabeled amino acid incorporation compared with control larvae.

yeast, it will be interesting to examine whether the stimulatory effects of increased Pol I activity on levels of Pol II- and III-dependent ribosome components are observed in mammalian cells.

Materials and methods

Fly stocks

UAS-TIF-IA transgenic lines were generated by cloning a full-length TIF-IA cDNA into the pUAST vector and transforming *w¹¹¹⁸* flies. The following other fly stocks were used: *ywshsFlp¹²²;+;Act>CD2>GAL4,UAS-GFP, da-GAL4, en-GAL4, UAS-Rheb* (Saucedo et al., 2003), *tor^d/CyO* (Zhang et al., 2000), UAS-S6K, and UAS-eIF4E. Flies were maintained on standard fly food. For protein starvation experiments, larvae were maintained on standard fly food for 2 d and then starved by floating on 20% sucrose/PBS solution. For rapamycin experiments, larvae were maintained on standard fly food for 2 d and then switched to food supplemented with either rapamycin or DMSO control.

S2 cell culture and double-stranded RNAi

RNAi experiments in S2 cells were performed as described previously (Hall et al., 2007). Flow cytometry was performed on S2 cells after 3 d of RNAi treatment as described previously (Hall et al., 2007).

Flow cytometry and clonal analysis in wing imaginal discs

The *hsFlp-Gal4* system was used to induce GFP-expressing cell clones in the larval wing imaginal disc at 72 h after egg deposition. At wandering,

flow cytometry was performed on dissociated imaginal wing disc cells as described previously (Grewal et al., 2005). Flow cytometry profiles were compared between GFP-expressing cell clones and non-GFP-expressing surrounding cells, which served as internal controls. To measure clone areas, GFP-marked cell clones were induced at 72 h after egg deposition, and wing discs were fixed and clone areas measured at wandering larval stage 3 (L3).

In situ hybridization and immunostaining

In situ hybridizations were performed as described previously, using digoxigenin-labeled probes to the external transcribed spacer (ETS) region of rRNA (Grewal et al., 2005). Immunostaining was performed as described previously (Grewal et al., 2005). The monoclonal antibody to fibrillarlin was used at a dilution of 1:500. All secondary antibodies used were Alexa Fluor 488 or 568 dye labeled (Invitrogen).

Microscopy

The images in Fig. 1 E were obtained on an optical sectioning microscope (DeltaVision RT; Applied Precision) consisting of a stand (IX-70; Olympus) fitted with a camera (Coolsnap HQ cooled CCD; Photometrics) and an oil immersion objective (UAPO 40 \times /1.35; Olympus). Images were deconvolved on a Linux workstation (Dell), using the SoftWoRx software package (Applied Precision). Other images were captured by a digital camera (DC480; Leica) and IM50 software (Leica) using either a stereomicroscope (MZ12; Leica; Fig. 1, B and C; and Fig. S1 A) or a microscope (DMRB; Leitz) with 40 \times objectives (Fig. 1, D and G; and Fig. 2, B, F, and G). Microscopy and image capture was done at room temperature and captured images were processed using Photoshop 7.0 (Adobe).

RNA analysis

All Northern blot experiments were performed according to the manufacturer's protocols (DIG nonradioactive nucleic acid labeling and detection kit; Roche). Total RNA was extracted from larvae using TRIzol (Invitrogen). For all experiments, equal numbers of similarly sized and developmentally staged larvae were used per experimental group. For pulse-chase labeling of rRNA, larvae were inverted in Ringer's solution and incubated in Ringer's containing 50 $\mu\text{Ci/ml}$ L-[methyl- ^3H]methionine (GE Healthcare) for 30 min. Larvae were then chased in Schneiders *D. melanogaster* media (Invitrogen) containing excess cold methionine. RNA were then analyzed by Northern blot. Blots were sprayed with EN3HANCE (PerkinElmer) and exposed to film.

Quantitative RT-PCR

Total RNA was extracted from larvae using TRIzol. For all experiments, equal numbers of similarly sized and developmentally staged larvae were used per experimental group. Reverse transcriptions and quantitative PCR were performed as previously described (Van Gilst et al., 2005). Levels of pre-rRNA were measured using primers to the ETS region of rRNA. Data were corrected for levels of either glyceral 3-phosphate dehydrogenase (GPDH) or dMyc mRNA, which were statistically unchanged between control and experimental groups. All data were analyzed by Student's *t* tests.

Ribosome subunit analysis

Control and TIF-IA-overexpressing larvae were lysed in a dissociating buffer lacking MgCl_2 . Lysates were loaded on 5–56% sucrose gradients, and ribosome subunits were separated by centrifugation at 38,000 rpm for 4 h at 4°C using a rotor (SW41; Beckman Coulter). Ribosome subunits were quantified by measuring the A260 of fractions collected from the gradients.

Protein synthesis assays

L3 larvae were inverted in Ringer's solution and incubated in Ringer's containing 15 $\mu\text{Ci/ml}$ of tritiated amino acid mix (GE Healthcare) at room temperature for 1 h. Carcasses were then washed in cold Ringer's and lysed. Equal amounts of protein per sample were then extracted from lysates using strataclean resin (Stratagene). Protein-bound resin was washed and transferred to scintillation buffer. 1-min counts were obtained with an LS6500 (Beckman Coulter).

DamID

Cell culture, transfections, and genomic DNA extraction and digestion were performed as described previously (Grewal et al., 2005). Cells were transfected with either hs-dam or hs-dam-TIF-IA. Cells were harvested at 48 h after transfection. All experiments were done in the absence of heat shock, allowing for low levels of dam and dam-TIF-IA expression through leaky transcription from the hs promoter. Methylation levels were measured by quantitative real-time PCR. Equal amounts of genomic DNA were digested with DpnII, which cuts nonmethylated DNA. Equal amounts of digested DNA were analyzed by quantitative PCR using primers that flank putative GATC-Dam methylation sites (nt 11,848–11,851 from GenBank/EMBL/DBJ under accession no. M21017; primers: left, AAACCGCAA-AGGCTCATT; right, GCACACGTCCCATAGGTTCC). Hence, the level of PCR signal gives an indication of methylation.

Online supplemental material

Fig. S1 shows that TIF-IA expression is sufficient to rescue *Tif-IA*^{-/-} mutant larvae to complete viability. Fig. S2 shows that rRNA processing is normal in TIF-IA-overexpressing larvae. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200709044/DC1>.

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