The Essential Drosophila ATP-binding Cassette Domain Protein, Pixie, Binds the 40 S Ribosome in an ATP-dependent Manner and Is Required for Translation Initiation*

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The Drosophila gene, pixie, is an essential gene required for normal growth and translation. Pixie is the fly ortholog of human RLI, which was first identified as an RNase L inhibitor, and yeast Rli1p, which has recently been shown to play a role in translation initiation and ribosome biogenesis. These proteins are all soluble ATP-binding cassette proteins with two N-terminal iron-sulfur clusters. Here we demonstrate that Pixie can be isolated from cells in complex with eukaryotic translation initiation factor 3 and ribosomal proteins of the small subunit. In addition, our analysis of polysome profiles reveals that double-stranded RNA interference-mediated depletion of Pixie results in an increase in empty 80 S ribosomes and a corresponding decrease in polysomes. Thus Pixie is required for normal levels of translation initiation. We also find that Pixie associates with the 40 S subunit on sucrose density gradients in an ATP-dependent manner. Our observations are consistent with Pixie playing a catalytic role in the assembly of complexes required for translation initiation. Thus, the function of this soluble ATP-binding cassette domain protein family in translation initiation has been conserved from yeast through to higher eukaryotes.

We recently carried out a genetic interaction screen to identify novel regulators of growth in Drosophila imaginal discs (1). Mutations in one of the isolated genes, pixie, have an unusual effect on fly growth that is similar to the effect of mutations in genes encoding ribosomal proteins. For example, weak pixie mutant combinations and some heterozygous ribosomal protein mutants have slender bristles and show developmental delay, yet their final adult body size can be near normal (2, 3). In addition, pixie and ribosomal protein mutant wing imaginal discs show complex defects in growth and cell survival that vary spatially and temporally (2). These and other observations suggest that the impact that mutating pixie has on growth might arise via an effect on translation. Indeed, dsRNAi2-mediated depletion of Pixie reduces global translation (2). To understand more about Pixie function and the way in which it affects translation, we have performed a biochemical analysis of its function.

Pixie is an exceptionally conserved protein found in all eukaryota and archaea sequenced so far (4). For example, Pixie and yeast Rli1p share 66% amino acid identity, and yeast Rli1p and human RLI have 67% amino acid identity. Pixie belongs to the ABC subfamily of ABC proteins, which contain two nucleotide-binding domains and two N-terminal iron-sulfur clusters. In contrast to most ABC domain proteins, members of this subfamily do not contain the membrane-spanning domains that would enable them to function as transporters (5). The human Pixie ortholog RLI, also known as ABCE1, was first characterized via its ability to inhibit the antiviral activity of RNase L (6–8). However, RNase L is only found in mammals, so this function of RLI does not account for its conservation in non-mammalian species (4). More recently, three independent studies have provided evidence that the yeast ortholog of Pixie, Rli1p, associates with translation initiation factors and the 40 S ribosomal subunit and plays a role in both translation initiation and ribosome biogenesis (9–11). Additional data suggest that the vertebrate and Caenorhabditis elegans RLI orthologs also play roles in translation (12–14). These fundamental functions are likely to explain the high conservation of the RLI/Pixie protein family and are consistent with the pixie mutant phenotypes that we have observed in Drosophila (2).

Translation initiation is a multistep process involving the sequential assembly of various large pre-initiation complexes. First, eIF1, eIF1A, eIF3, eIF5 and the ternary complex, which consists of eIF2 bound to GTP and the initiator tRNA^Met, are recruited to the small 40 S ribosomal subunit to form the 43 S pre-initiation complex. Then, the 40 S subunit binds to the capped 5′-end of mRNAs in a reaction requiring eIF4F, eIF4A, and eIF4B. This complex scans the mRNA and positions itself on the correct initiation AUG codon, thereby forming the 48 S pre-initiation complex. Finally, the eIFs are displaced and the large 60 S ribosomal subunit is recruited in an eIF5B-dependent reaction to generate functional 80 S ribosomes (15, 16).

* This work was supported by Cancer Research UK and the Danish Harboe Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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2 The abbreviations used are: dsRNAi, double-stranded RNA interference; ABC, ATP-binding cassette; eIF, eukaryotic translation initiation factor; EAHX, aminohexanoic acid; CB, cleavage buffer; CaM, calmodulin; CaMBB, calmodulin binding buffer; GST, glutathione S-transferase; TEV, tobacco etch virus; CBP, calmodulin-binding peptide; GTC, GST-TEV-CBP; PDVF, polyvinylidene difluoride; GFP, green fluorescent protein; RpLP, ribosome P antigen; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; PBS, phosphate-buffered saline; Bis-Tris, 2-(bis[2-hydroxyethyl]amino)-2-(hydroxymethyl)propane-1,3-diol; ADP-N-P, non-hydrolyzable version of ATP.
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elf3 is thought to have multiple functions in translation initiation (17). elf3 can bind the 40 S subunit independently of other elfs, and several studies in vitro and in yeast have implicated elf3 in promoting the recruitment of the ternary complex to the 40 S subunit to form the 43 S pre-initiation complex (15, 16). In addition, a multifactor complex consisting of elf1, elf3, elf5, and the ternary complex has been isolated, which is likely to provide an alternative route for the assembly of the 43 S pre-initiation complex (18). Finally, upon termination of translation, elf3 is thought to inhibit the assembly of the released 40 S and 60 S subunits into empty 80 S ribosomes lacking mRNA. The association of elf3 with the 40 S subunit alone does not prevent premature 40 S-60 S subunit assembly, but simultaneous binding of elf3 and the ternary complex to the small ribosomal subunit does prevent the recruitment of the 60 S ribosomal subunit in the absence of mRNA (16, 19, 20).

elf3 has a complex multisubunit structure. All eukaryotes possess five core elf3 components: Tif32p/elf3-S10/elf3a, Nip1p/elf3-S8/elf3b, Prt1p/elf3-S9/elf3c, Tif35p/elf3-S4/elf3g, and Tif34p/elf3-S2/elf3i, and one loosely associated component, Hcr1p/Adam/elf3j (yeast/flies/humans) (17). Mammalian elf3 has been shown to contain at least seven additional subunits. Although not previously studied, Drosophila has orthologs of all 13 mammalian elf3 components, indicating that the architecture of fly elf3 resembles that of mammalian rather than yeast elf3 (21, 22).

As was mentioned above, the yeast ortholog of Pixie, Rli1p, was recently shown to co-purify with various yeast translation initiation factors, including elf3 (9, 10). However, a proportion of the core components of yeast elf3 is consistently found associated with the 40 S ribosomal subunit, whereas Rli1p was only detected associated with the 40 S subunit under low salt conditions or after cross-linking (9–11). In addition, Rli1p is less abundant than other elf3 components (9). Together these observations suggest that Rli1p is not a core component of elf3 and that the binding of Rli1p to the 40 S subunit may be dynamic or regulated (11). In addition to its role in translation initiation, Rli1p has also been shown to play a role in processing or after cross-linking (9–11). In addition, Rli1p is less abundant than other elf3 components (9). Together these observations suggest that Rli1p is not a core component of elf3 and that the binding of Rli1p to the 40 S subunit may be dynamic or regulated (11). In addition to its role in translation initiation, Rli1p has also been shown to play a role in processing of the small and large ribosomal subunits (9–11).

To characterize the biochemical function of Pixie, we first carried out a tandem affinity purification of recombinant Pixie from cultured Drosophila cells. Like Rli1p, Pixie associates with core components of elf3 and with ribosomal proteins. Furthermore, depletion of Pixie results in increased numbers of monosomes and fewer polysomes, indicating that Pixie is required for normal levels of translation initiation. These observations led us to characterize more closely Drosophila elf3 and the role of Pixie and Drosophila elf3 in translation initiation. We found that, in addition to associating with the 40 S ribosome, the core components of Drosophila elf3 are found in a high molecular weight complex that may represent elf3 itself or the Drosophila equivalent of yeast multifactor complex. Whereas a proportion of elf3 is consistently found associated with the 40 S subunit, the association of Pixie with the 40 S ribosome is dynamic and regulated via its ability to bind ATP. Furthermore, Pixie can associate with the 40 S subunit independently of its interaction with elf3. Together, our data demonstrate that Pixie interacts with elf3 and the 40 S ribosome and is required for translation initiation in Drosophila.

MATERIALS AND METHODS

Cell Culture—Drosophila S2 cells were grown in Schneider medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Sigma), 50 units/ml penicillin, and 50 μg/ml streptomycin (complete medium, Invitrogen) at 25 °C. S2 cells were transfected with pDA9-1, pDA9-2, and pDA10-1 (see plasmids below) using a calcium phosphate transfection kit (Invitrogen) or with pDA9-3 using an Effectene transfection kit (Qiagen). Cells stably expressing GTC-Pixie, GTC-PixieABC, GTC-PixieE501Q, or the GTC tag alone were selected in medium containing 300 μg/ml hygromycin. For insulin treatment cells were treated with 1 μM bovine insulin in complete medium.

Antibodies—Polyclonal rabbit antisera were raised against the Adam (2T) N-terminal peptide: MADDWESADSEVIRP-Cys, the elf3-S10 (5T) N-terminal peptide: MARYTQR-PENALKRANEFIAEHX-Cys (where EAHX corresponds to the aminohexanoic acid spacer), the elf3-S2 (CT1) C-terminal peptide: Cys-EAHX-RVQTFDSTYFENIFE, the elf3-S8 (CT2) C-terminal peptide: C-EAHX-KNNQQQQVQTVTI-DEE, the elf3-S9 (CT1) C-terminal peptide: C-EAHX-DEVDEE-IVEFLVKEE, and the Rps25 (NT2 or NT1) N-terminal peptide: MPPKDASSAKQPQKTOQKK-EAHX-Cys. All peptides were coupled via their N- or C-terminal cysteines to maleimide-activated keyhole limpet hemocyanin (Pierce) as directed. Polyclonal rabbit antisera were raised against the elf-2α (2396) N- and C-terminal peptides: SRFYNERYPEIDVC and CQFPDKEFENHKSGA, and the Rps13 (I2) internal peptide: YYKTKSVLPFNWKYE (Eurogentec).

Plasmids—The plasmids to express recombinant Pixie or PixieABC tagged at the N terminus with glutathione S-transferase (GST) followed by a tobacco etch virus (TEV) protease cleavage site and a calmodulin-binding peptide (CBP) (collectively referred to as “GTC”) was generated in three steps. First, the entire Pixie open reading frame or Pixie lacking the second ABC domain (PixieABC), with EcoRV, HindIII, and EcoRI sites inserted before the start codon and with a KpnI site inserted after the start codon was PCR-amplified using the primers 5′-CGATATCCGGAAGCTTGAATTCGCCACCATGGAATCTCAGC-3′ and 5′-GGCGGGCGCCCTAGTTTACATCGAATGACGT-3′ (Pixie) or 5′-CGCGGGCGCCGTAGATACCCTCGC-3′ (Pixie) and 5′-GGCGGGCGCCGTAGATACCCTCGC-3′ (PixieABC). The PCR fragments were inserted into pGEM-T (Promega, Madison, WI) giving rise to the pDA3-1 and pDA3-3 plasmids, and the insert DNA sequences were verified. Second, a cassette encoding the GTC tag (23) was subcloned from pCN-GTC tag (gift from Pascal Meier) into pDA3-1 and pDA3-3 using the HindIII and EcoRI restriction sites, thereby generating pDA8-1 and pDA8-2, respectively. Third, N-terminally GTC-tagged Pixie and PixieABC were inserted in front of the inducible metallothionein promoter of the pMK33 vector using the unique EcoRV and SpeI restriction sites to generate pDA9-1 and pDA9-2, respectively. GTC-PixieE501Q (pDA9-3) was generated by introducing a point mutation in the second ABC domain of Pixie (G1483C) by three consecutive PCRs.
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PCR fragment was amplified using the primers 5′-CCGGTAAC-CTCCACCGATGG-3′ and 5′-ACTAGTGGCGCCGCTTTAGTTGAGGTTCTGGTCTTCCA-3′, which covers a unique BstEII restriction site 5′ of the point mutation, and inserts a SpeI site 3′ of the stop codon, respectively. This PCR fragment was inserted into pCR®2.1-TOPO® (Invitrogen) generating pDA3-5, and the DNA sequence was verified. Finally, the Pixie fragment containing the point mutation was subcloned into pDA9-1 using the unique BstEII and SpeI restriction sites to generate pDA9-3.

A control plasmid expressing the GTC tag alone was generated by PCR amplification from the pCN-GTC tag using primers that added a EcoRV and SpeI site in front of the ATG and after the stop codon, respectively. This fragment was subcloned into pMK33 to generate pDA10-1.

**Tandem Affinity Purification from S2 Cells Expressing Recombinant Pixie**—Two-step purifications were performed from 1 × 10^10 S2 cells expressing recombinant GTC-Pixie or the GTC tag alone. Cells were lysed in 50 ml of Buffer A (50 mM Heps-KOH, pH 8, 100 mM KCl, 10 mM MgCl₂, 0.1% Triton X-100, 2 mM dithiothreitol, 10% glycerol, 10 mM NaF, 0.25 mM NaVO₄, 1 mM phenylmethylsulfonyl fluoride, 0.1 µg/ml aprotonin, 10 µg/ml leupeptin), and cell extracts were cleared of nuclear and membranous material by centrifugation at 10,000 rpm for 15 min. In the first purification step, the cleared cell extracts were incubated with glutathione beads (Sepharose-4B, Amersham Biosciences) for 2 h at 4°C. Post incubation, beads were washed three times in 12 ml of Buffer A and three times in Cleavage Buffer (CB; 10 mM Tris-HCl, pH 8, 150 mM NaCl, 0.1% Tween 20) and incubated overnight at 4°C in Cleavage Buffer (CB; 10 mM Tris-HCl, pH 8, 150 mM NaCl, 0.1% Tween 20) or PBS-T (137 mM NaCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, 2.7 mM KCl, 0.1% Tween 20, pH 7.4) then probed with secondary antibodies labeled with Alexa Fluor 680 (Molecular Probes) or IRDye™ 800 (Rockland Immunocchemicals) diluted 1/6000 in a buffer containing one-third volume of TBS-T or PBS-T, two-thirds volume blocking buffer for 1 h at room temperature. After washing in TBS-T or PBS-T, bands were detected using the Odyssey Infrared Imaging System (LI-COR Biosciences).

**Sucrose Gradient Fractionation of S2 Cell Extracts**—To generate the data shown in Figs. 4–7, 5 × 10⁸ S2 cells were treated with 100 µg/ml cycloheximide (Sigma) for 10 min and lysed in 0.6 ml of 50 mM Heps-KOH, pH 7.2, 100 mM KCl, 10 mM MgCl₂, 0.1% Triton X-100, 100 µg/ml heparin, 2 mM dithiothreitol, 100 µg/ml cycloheximide, and RNAGuard™ (Amersham Biosciences). Cell debris was removed via centrifugation for 10 min at 13,000 rpm, and extracts were resolved on 7.5–30% sucrose density gradients by centrifugation for 4.5 h at 39,000 rpm at 4°C using an SW41Ti rotor (Beckman). 600-µl fractions were collected while recording the A₂₅₄ profile using a single path UV monitor (Amersham Biosciences).

To generate the data shown in Fig. 3, S2 cells were seeded in 35-mm wells at a density of 7 × 10⁵ cells/ml in a total of 3 ml of complete medium/well and treated with dsRNA (25 µg/well) targeting GFP (control), Pixie, or eIF3-S10, for 7–8 (Pixie) or 5 (eIF3-S10) days, and the effectiveness of the dsRNA-mediated depletion was assessed by immunoblotting. To stimulate growth, cells were starved for 1 h in PBS containing 2 mg/ml glucose (Sigma) and subsequently stimulated in complete media containing 1 µg insulin (Sigma) for 3 h. Cells were then treated with 100 µg/ml cycloheximide (Sigma) for 10 min, and cytoplasmic extracts were prepared from 3 × 10⁶ cells as described above. The cleared extracts were resolved on 7.5–60% sucrose density gradients by centrifugation at 39,000 rpm for 2 h at 4°C using a SW41Ti rotor (Beckman). The A₂₅₄ profiles were recorded as described above.

**dsRNA**—dsRNA was prepared as described (2). Primers contained 5′-T7 RNA polymerase-binding sites preceded by a GAA overhang and followed by sense or antisense sequences. For eIF3-S10, sense primer: 5′-TGTTCTGGAGCTGTAGTAC-3′, antisense primer: 5′-GTAGTTGGCCATGTGTTTGG-3′, for GFP, sense primer: 5′-GGTGTTGCCCATTCTGTGGT-3′, antisense primer: 5′-TGCCGCTTCGTGGGCGG-3′, for pS25, sense primer: 5′-ATTCAAGTGCAAAGTGTATATAC-3′ and 5′-TTGGCAACATTCCAGCGGTAAAGA-3′, antisense primers: 5′-TGTTAGTAGCTGGAGATTGTG-3′ and 5′-CTTGGTGGCACTGTGTA-3′, and for pS13, sense primer: 3′-CGTTGACCAAGCAGG-3′, antisense primer: 5′-GCAGTGCTGACATCGTA-3′.

**RESULTS**

**Pixie Associates with eIF3 and Ribosomal Proteins**—We used a tandem affinity purification method to identify Pixie-associ-
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FIGURE 1. Pixie associates with eIF3 and ribosomal proteins. A, schematic of GTC-Pixie. B, anti-Pixie immunoblot of extracts prepared from S2 cells expressing GTC-Pixie under the control of the inducible metallothionein promoter, incubated for 16 h with increasing concentrations of CuSO4. C and D, a two-step purification was performed from \( \sim 1 \times 10^{10} \) S2 cells expressing GTC-Pixie or the GTC tag alone. The final eluates were resolved on a 4–12% Nu-PAGE Bis-Tris gel and stained with Brilliant Blue G-colloidal concentrate (C) or blotted onto a polyvinylidene difluoride membrane and probed with antibodies against various translation initiation factors (D). Visible bands were excised and identified by MALDI-TOF mass spectrometry (marked with an asterisk in C), have sizes that correspond approximately to other eIF3 components, eIF3-S9 and eIF3-S8.

FIGURE 2. Maximal depletion of Pixie reduces the levels of some eIF3 components. Cell lysates were prepared from S2 cells treated with dsRNA corresponding to GFP (control) or Pixie for 5 days and analyzed by immunoblotting with the antibodies indicated. Depletion of Pixie causes a reduction in protein levels of eIF3-S8, eIF3-S9, and eIF3-S10, but not Adam or eIF3-S2.

Pixie Binds eIF3 and Functions in Translation Initiation—Although we have previously shown that Pixie is required for normal levels of translation (2), the way in which it influences translation has not been established. The observation that Pixie can be isolated in complex with several eIF3 components and ribosomal proteins from the small ribosomal subunit, Rap1 and Rps25, which repeatedly co-purified with GTC-affinity-purified Pixie (Fig. 1C). The final Pixie-associated protein identified by mass spectrometry, CG4975, has no identifiable domains and is found only in flies, so it was not studied further. To summarize, by taking a tandem affinity purification approach, we have shown that a metazoan Rli1p ortholog, Drosophila Pixie, associates with eIF3 and ribosomal proteins, consistent with the idea that the role of this family of soluble ABC proteins in translation initiation is conserved from yeast through to Metazoa.

Depletion of Pixie Impairs Translation Initiation—Although we have previously shown that Pixie is required for normal levels of translation (2), the way in which it influences translation has not been established. The observation that Pixie can be isolated in complex with several eIF3 components and ribosomal proteins from the small subunit suggests that, like its yeast and vertebrate orthologs, Pixie may have a role in translation initiation. To test this hypothesis, S2 cells were treated with double-stranded RNA (dsRNA) corresponding to green fluorescent protein (GFP, control) or Pixie, and the effect on polyribosome profiles was examined. Immunoblotting was used to confirm the dsRNAi-mediated depletion of Pixie and study its impact on eIF3 expression. Interestingly, we found that, when Pixie depletion was particularly effective, the levels of the core eIF3 components, eIF3-S8, eIF3-S9 and eIF3-S10, were also reduced (Fig. 2). In contrast, levels of eIF3-S2 and Adam were unaffected. This observation raises the possibility that Pixie plays a role in stabilizing core components of eIF3, perhaps by...
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Pixie Binds eIF3 and Functions in Translation Initiation. S2 cells were treated with dsRNA corresponding to GFP (control), Pixie or eIF3-S10 for five (D–F), or eight (A–C and G–I) days. Prior to harvesting, cells were starved in PBS containing 2 mg/ml glucose for 1 h, then stimulated with 1 μM insulin in complete Drosophila Schneider medium for 2 h, followed by treatment with 100 μg/ml cycloheximide for 10 min. A, B, D, E, G, and H, cytoplasmic extracts were resolved by velocity sedimentation on 7.5–60% sucrose gradients for 2 h at 39,000 rpm, then the A254 profile was recorded for each gradient. G and H, cytoplasmic extracts were resolved by velocity sedimentation in the presence of 0.7 M NaCl. The positions of different ribosomal species on the polysome profiles are indicated (A, G, and H), C, F, and I, an aliquot of each cytoplasmic extract was resolved by SDS-PAGE, and analyzed by immunoblotting.

Promoting their assembly into translation initiation-competent complexes. Thus, for the polysome profile experiment, care was taken to use an intermediate dsRNAi regime that did not impact upon core eIF3 component levels so that any changes in the polysome profile could be attributed specifically to Pixie rather than eIF3 depletion (Fig. 3C and data not shown).

Cytoplasmic extracts were resolved on 7.5–60% sucrose gradients, and their A254 profiles were recorded to generate polysome profiles on which peaks corresponding to the 40 S and 60 S ribosomal subunits, 80 S ribosomes, and polysomes could be easily distinguished (Fig. 3, A, B, D, E, G, and H). Partial depletion of Pixie (in the absence of any detectable eIF3 depletion) caused a reproducible decrease in polysome content and increase in 80 S ribosome content. The polysome:monosome ratio decreased from 2.6 in control cells to 1.0 in Pixie-depleted cells (Fig. 3, A and B). A similar effect was observed upon dsRNAi-mediated depletion of eIF3-S10 (Fig. 3, D–F).

The observed increase in the 80 S ribosome peak in cells depleted of Pixie or eIF3-S10 might indicate an increase in either stalled 80 S ribosomes bound to mRNA, empty 80 S complexes, or both. To distinguish between these possibilities, polysome profiles were resolved under high salt conditions to dissociate empty but not mRNA-bound ribosomes (24). Under these conditions, the 80 S peak in cells depleted of Pixie was greatly reduced and similar in size to that in control cells. Furthermore, there was a dramatic increase in the amount of free 40 S and 60 S subunits in cells depleted of Pixie (Fig. 3, G and H). Thus, Pixie is required for the formation of mRNA-bound ribosomes.

Pixie Does Not Behave Like a Core Component of eIF3—As recombinant GTC-Pixie can be affinity purified with eIF3 and small ribosomal subunit proteins, we next examined the association of endogenous Pixie and various eIF3 components with the translation machinery. S2 cell lysates were resolved on sucrose gradients, and fractions were collected and analyzed by immunoblotting. Initial analysis of the distribution of Pixie on 7.5–60% sucrose gradients revealed that Pixie is present in the low density fractions at the top of the gradient and possibly in the fractions containing the 40 S subunit. In contrast, no Pixie was found in the fractions containing the 60 S subunits, 80 S ribosomes, or polysomes (data not shown).

To increase resolution around the 40 S subunit, the experiment was repeated using a 7.5–30% sucrose gradient. Immunoblotting with antibodies against RpS25 and the ribosome P antigen (termed “RpLP”) identified the fractions containing the small and large ribosomal subunits, respectively (Fig. 4). As expected given their role in translation initiation, a proportion of the core eIF3 components, eIF3-S2, eIF3-S8, eIF3-S9, and eIF3-S10, is associated with the 40 S subunit. Notably though, the majority of these eIF3 proteins exists in less dense complexes that are slightly higher up on the gradient than the small ribosomal subunit (asterisk in Fig. 4). These complexes may correspond to the eIF3 complex itself or to the Drosophila equivalent of the yeast translation initiation intermediate, multifactor complex (18). Consistent with this latter possibility, another component of the multifactor complex, eIF2α, was also found in these fractions (Fig. 4).

FIGURE 3. Partial depletion of Pixie does not affect eIF3 levels, but impairs translation initiation. S2 cells were treated with dsRNA corresponding to GFP (control), Pixie or eIF3-S10 for five (D–F), or eight (A–C and G–I) days. Prior to harvesting, cells were starved in PBS containing 2 mg/ml glucose for 1 h, then stimulated with 1 μM insulin in complete Drosophila Schneider medium for 2 h, followed by treatment with 100 μg/ml cycloheximide for 10 min. A, B, D, E, G, and H, cytoplasmic extracts were resolved by velocity sedimentation on 7.5–60% sucrose gradients for 2 h at 39,000 rpm, then the A254 profile was recorded for each gradient. G and H, cytoplasmic extracts were resolved by velocity sedimentation in the presence of 0.7 M NaCl. The positions of different ribosomal species on the polysome profiles are indicated (A, G, and H), C, F, and I, an aliquot of each cytoplasmic extract was resolved by SDS-PAGE, and analyzed by immunoblotting.

FIGURE 4. eIF3, but not Pixie, associates with the 40 S subunit under standard conditions. Cytoplasmic extracts were prepared from 5 × 106 S2 cells and resolved by velocity sedimentation on 7.5–30% sucrose gradients for 4.5 h at 39,000 rpm. Gradient fractions were collected, and polysome profiles were simultaneously recorded (data not shown). Fractions were resolved by SDS-PAGE and analyzed by immunoblotting. The presence of the 40 S and 60 S ribosomal subunits was revealed by the A254 profile (not shown) and by probing for RpS25 and RpLP, respectively. The asterisk indicates an eIF3-containing complex that may correspond to the Drosophila equivalent of the yeast multifactor complex.
Pixie Associates with the 40 S Ribosome Subunit in an ATP-dependent Manner—We next considered the possibility that the ability of Pixie to interact with other proteins might be regulated via its ABC domains, which are predicted to bind and hydrolyze ATP. Consistent with these domains being important for Pixie function, several strong loss-of-function mutations in pixie are in conserved residues of the ABC domains (2). For example, pixie<sup>E<sub>C2</sub></sup> (Gln-231 → Leu) disrupts the nucleotide-binding ABC domain signature motif of the first ABC domain, and pixie<sup>3C2</sup> (Gly-123 → Ser) is in the third conserved glycine of the Walker A loop of the first ABC domain, which is predicted to bind to the nucleotide phosphate groups (2). Moreover, a mutation in yeast Rli1p that is predicted to allow ATP binding, but not hydrolysis, is non-functional and inhibits growth when overexpressed (9).

To investigate whether the association of Pixie with the 40 S subunit might be regulated by ATP binding and/or hydrolysis, ADP, ATP, or a non-hydrolyzable version of ATP (ADP-N-P) was added to S2 cell extracts prior to their resolution on 7.5–30% sucrose gradients. Immunoblotting showed that addition of ADP, ATP, or ADP-N-P to these extracts did not dramatically alter the distribution of eIF3-S2, eIF3-S8, eIF3-S9, and eIF3-S10, RpLP, or RpS25 on the sucrose gradients (Fig. 5A). Similarly, the addition of ADP or ATP had no effect on the distribution of Pixie on the sucrose gradients. Importantly, we consistently found that adding non-hydrolyzable ADP-N-P to cell extracts caused the redistribution of some Pixie into the fractions containing the 40 S subunit (Fig. 5A). Given the similar distribution of Pixie and Adam on the initial sucrose gradients (Fig. 4), we also examined the effect of ADP-N-P on the distribution of Adam. However, no consistent redistribution of Adam was observed (data not shown).

To ensure that the presence of Pixie in these fractions was due to an association with the 40 S subunit rather than another dense protein complex, dsRNAi was used to deplete cells of RpS13 and Rps25 and hence the 40 S subunit (Fig. 5C and data not shown). Under these conditions addition of ADP-N-P was unable to redistribute Pixie to the denser fractions of the sucrose gradient (Fig. 5B). Thus, the appearance of Pixie in these fractions does indeed require its association with the 40 S subunit.

We next sought to examine whether it is the binding of ATP to Pixie itself that allows its association with the 40 S subunit. We generated cells stably expressing a GTC-tagged Pixie deletion mutant lacking the second ABC domain (PixieΔABC). The distribution of GTC-PixieΔABC on sucrose gradients was examined in the presence of ADP-N-P. Unlike endogenous Pixie and GTC-Pixie, GTC-PixieΔABC did not bind to the 40 S in the presence of ADP-N-P (Fig. 6A).

Next cells were generated that stably express a mutated version of Pixie in which glutamate 501 in the conserved Walker B motif of the second ABC domain was replaced with glutamine (PixieE501Q). This mutation is predicted to allow ATP binding but not hydrolysis; the analogous mutation in yeast makes Rli1p biologically inactive (9). On sucrose gradients, GTC-PixieE501Q, unlike wild-type Pixie, was found in the fractions containing the 40 S subunit (Fig. 6B). In fact its distribution was very similar to that of other elf3 components, e.g. eIF3-S10 (Figs. 4 and 6B).
was used to deplete S2 cells of the eIF3 core component eIF3-S10, and the levels of 40 S-bound Pixie in the presence of ADP-N-P were examined (Fig. 7A). In addition to significantly depleting eIF3-S10 protein levels (Fig. 7A, upper panels), eIF3-S10 dsRNAi also reduced the protein levels of the core eIF3 components, eIF3-S8 and eIF3-S9, though it had no effect on the protein levels of eIF3-S2, Adam, or Pixie (Fig. 7B and data not shown). This observation is consistent with the idea that eIF3 core components co-stabilize each other (see Fig. 2 and accompanying text). Notably, reducing the levels of 40 S-bound eIF3-S10, eIF3-S8, and eIF3-S9 had no effect on the ability of Pixie to bind to the small ribosomal subunit (Fig. 7A, compare Pixie distribution in ADP-N-P lanes with ADP-N-P + dsRNA-S10 lanes). Although it is possible that the small amount of remaining eIF3-S10 was responsible for the association of Pixie with the 40 S subunit, the fact that there was no detectable reduction in 40 S-bound Pixie strongly implies that the ability of Pixie to bind the 40 S subunit is independent of its ability to interact with eIF3. Note that levels of the non-core eIF3 components Adam and eIF3-S2 were unaffected by eIF-S10 depletion (data not shown), so these proteins may bridge the interaction between Pixie and the 40 S subunit. Indeed, the mammalian homolog of Adam, eIF3j, can associate with the 40 S subunit independent of its interaction eIF3.

**DISCUSSION**

Here we show that *Drosophila* Pixie can be purified in complex with eIF3 components (Fig. 1) and that, like depletion of the eIF3 core component, eIF3-S10, depletion of Pixie results in the accumulation of empty 80 S ribosomes and a decrease in polysomes (Fig. 3). Thus, like its yeast and vertebrate orthologs, Rli1p and ABCE1, Pixie is required for normal levels of translation initiation (9–12).

We also noticed that extensive dsRNAi-mediated depletion of Pixie caused a reduction in the protein levels of three core eIF3 components, eIF3-S8, eIF3-S9, and eIF3-S10, whereas levels of eIF3-S2 and Adam were unaffected (Fig. 2E). In addition, dsRNAi-mediated depletion of eIF3-S10 reduced levels of eIF3-S8 and eIF3-S9 but not Adam and Pixie (Fig. 6 and data not shown). These observations suggest that the optimal stability of eIF3-S8, eIF3-S9, and eIF3-S10 depends on the presence of all three proteins in stoichiometric amounts and their assembly into complexes. Furthermore, Pixie may aid the assembly of these eIF3 components into stable translation initiation competent complexes. In contrast, depletion of yeast Rli1p had no effect on the protein levels of Prt1p/eIF3-S9 nor the protein levels of eIF2 and eIF5 (9). Thus, some aspects of Pixie and Rli1p function may not be conserved between flies and yeast.

In addition to its association with eIF3, we found that affinity-purified Pixie consistently associates with two small ribosomal proteins, RpS17 and RpS25, raising the possibility that Pixie can bind to the 40 S subunit under certain conditions. By examining the distribution of endogenous Pixie on sucrose gradients, we found that Pixie can associate with the 40 S ribosomal subunit, but that this association can only be detected in the presence of a non-hydrolyzable version of ATP (ADP-N-P) and not ATP or ADP (Fig. 5C). Pixie possesses two ABC domains, the integrity of which is essential for its biological function (2).
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Thus, the observed association of Pixie with the 40 S subunit in the presence of ADP-N-P may be a direct result of the ability of Pixie itself to bind and hydrolyze ATP. Consistent with this hypothesis, a mutated version of Pixie that is predicted to bind but not hydrolyze ATP is constitutively bound to the 40 S subunit (Fig. 6).

Given the fact that Pixie can bind both translation initiation factors and ribosomal proteins, and the effect that ADP-N-P has on its distribution on sucrose gradients, Pixie may play a catalytic role in the assembly of translation pre-initiation complexes onto the 40 S subunit. Based on the low abundance of Rli1p relative to eIF2, eIF3, and eIF5, Dong et al. (9) have also speculated that yeast Rli1p plays a catalytic role in the assembly of pre-initiation complexes on the 40 S ribosome rather than acting as a stoichiometric component of these complexes. Consistent with Pixie having a catalytic role and with the interactions between Pixie and eIF3 and the 40 S subunit being dynamic, others have found that the association of Rli1p with eIF3 and the 40 S is only detectable under low salt conditions (10, 11) or after covalent cross-linking (9). To investigate whether Pixie plays a role in the recruitment of eIF3 to the 40 S subunit, we compared levels of 40 S-bound eIF3-S10 in cells depleted of Pixie with that in control cells. However, we found that a 90% depletion of Pixie did not affect levels of 40 S-bound eIF3-S10 or eIF2 (data not shown), either because Pixie is not essential for their recruitment to the 40 S subunit, or because the remaining Pixie is still able to provide that catalytic function. Notably though, the same 90% depletion of Pixie was able to reduce the polysome content of cells (Fig. 3), indicating that this observed defect in translation initiation is unlikely to be caused by a lack of recruitment of eIF3 or eIF2 to the 40 S subunit. Thus, Pixie may play a role in a later step of translation initiation, between the assembly of the 43 S pre-initiation complex and the 48 S pre-initiation complex.

Our previous phenotypic analyses have shown that the pixie mutant phenotype is similar to that displayed by the Minute class of mutations, several of which have been shown to correspond to mutations in ribosomal genes (see introduction and Ref. 3). Like flies heterozygous for Minute mutations, flies trans-heterozygous for weak pixie mutant combinations, or flies carrying a dominant negative pixie allele show developmental delay and have slender bristles (2). Thus, pixie has a "Minute-like" phenotype, consistent with it being required for normal levels of translation. To date, no translation initiation factors have been reported to have Minute-like phenotypes, and eIF4A mutations do not have the same effect on clonal growth as pixie mutations (2). Thus, although our biochemical data demonstrate that Pixie plays a role in translation initiation, its mutant phenotypes are more like those of ribosomal protein mutants than those of translation initiation factors. The Minute-like aspects of the pixie mutant phenotype may reflect the fact that, like yeast Rli1p, Pixie has an additional role in ribosome biogenesis (9–11). Consistent with this idea, our data demonstrate that Pixie can bind the 40 S ribosomal subunit (Fig. 1C) and that it does so independently of core eIF3 components (Fig. 6A). Notably though, we were unable to detect any difference in Rps25 levels by immunoblotting cells depleted of Pixie (data not shown), much like Kispal et al. (11), who saw no reduction in Rps3 and Rpl10 levels following Rli1p depletion. Furthermore, when Pixie-depleted extracts were treated with high salt, there was no detectable difference in the absolute levels of free 40 S and 60 S subunits compared with control cells (data not shown).

The data discussed here demonstrate that the reported role of Rli1p in translation initiation is conserved in multicellular eukaryotes, whereas its function in ribosome biogenesis might be conserved. These fundamental functions may well be the essential functions that account for the high degree of conservation of this protein family. As was discussed above, the ABC domains of Pixie and Rli1p are essential for their in vivo function. Additional data suggest that the same is true of the highly conserved iron-sulfur clusters at the N terminus of these proteins. For example, in the strong loss-of-function mutation, pixie<sup>17</sup>, a converted alanine of the second iron-sulfur cluster is mutated to threonine. Furthermore, the integrity of both Rli1p iron-sulfur clusters is essential for yeast viability (2, 11). The function of the Pixie/Rli1p iron-sulfur clusters remains to be determined, but it is tempting to speculate that input via the iron-sulfur clusters may regulate the activity of the ABC domains, for example in response to changing environmental conditions.
conditions such as increased levels of reactive oxygen species (11).

Acknowledgments—We thank Pascal Meier and Tencho Tenev for providing the pcN-GTC tag vector, Terence Herbert, Isabel C. Greenman, and Philip Whitehead for advice and assistance with polysome profile analysis, and Chris Proud for anti-eIF4A antibodies. We also thank the Cancer Research UK London Research Institute Equipment Park and Fly Facility for technical support, the Cell Production service for large scale culturing of S2 cells, the Protein Identification laboratory for Mass Spectrometry analysis, and the Peptide Synthesis laboratory for generating peptides for antisera production. We are grateful to Nicolas Tapon for many helpful discussions, and Steven Marygold and Nicolas Tapon for critical reading of the manuscript.

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