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The *Drosophila* EKC/KEOPS complex

Roles in protein synthesis homeostasis and animal growth

Diego Rojas-Benítez, Consuelo Ibar and Álvaro Glavic*

FONDAP Center for Genome Regulation; Departamento de Biología; Facultad de Ciencias; Universidad de Chile; Santiago, Chile

The TOR signaling pathway is crucial in the translation of nutritional inputs into the protein synthesis machinery regulation, allowing animal growth. We recently identified the Bud32 (yeast)/PRPK (human) ortholog in *Drosophila*, Prpk (p53-related protein kinase), and found that it is required for TOR kinase activity. Bud32/PRPK is an ancient and atypical kinase conserved in evolution from *Archeae* to humans, being essential for *Archeae*. It has been linked with p53 stabilization in human cell culture and its absence in yeast causes a slow-growth phenotype. This protein has been associated to KEOPS (kinase, putative endopeptidase and other proteins of small size) complex together with Kae1p (ATPase), Cgi-121 and Pcc1p. This complex has been implicated in telomere maintenance, transcriptional regulation, bud site selection and chemical modification of tRNAs (tRNAs). Bud32p and Kae1p have been related with N6-threonylcarbamoyladenosine (t^6A) synthesis, a particular chemical modification that occurs at position 37 of tRNAs that pair A-starting codons, required for proper translation in most species. Lack of this modification causes mistranslations and open reading frame shifts in yeast. The core constituents of the KEOPS complex are present in *Drosophila*, but their physical interaction has not been reported yet. Here, we present a review of the findings regarding the function of this complex in different organisms and new evidence that extends our recent observations of Prpk function in animal growth showing that depletion of Kae1 or Prpk, in accordance with their role in translation in yeast, is able to induce the unfolded protein response (UPR) in *Drosophila*. We suggest that EKC/KEOPS complex could be integrating t^6A-modified tRNA availability with translational rates, which are ultimately reflected in animal growth.

Keywords: growth, tRNA modification, translation, TOR signaling, PRPK, Kae1

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*Correspondence to: Álvaro Glavic;
Email: alglavic@uchile.cl

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Bud32/PRPK is a Conserved Atypical Kinase

Bud32 was first identified in yeast as a putative kinase with atypical features.[1] This protein is an extremely small acidophilic kinase conserved through evolution from A*rcheae* to *Eukarya*. Orthologs have been identified and studied in *Methanocaldococcus jannaschii*, *Haloferax volcanii*,[2,3] *Saccharomyces cerevisiae* (yeast),[1,4,5] mammals[6,7] and recently by our group in *Drosophila melanogaster*.[8] This protein is essential in *Archeae*,[2,3] while, in yeast, Δ*bud32* mutant has a slow-growth phenotype with reduced cell division rate and achieves a low concentration at stationary phase.[4]

In mammals, the Bud32 ortholog, named PRPK (p53-related protein kinase), was first identified as a transcript enriched in IL-2-activated cytotoxic T cells, testis and tumor-derived epithelial cells.[9] Human PRPK is capable to partially complement the Δ*bud32* mutant yeast, indicating its functional conservation.[7] Previous studies have reported its kinase activity on acidic target and its ability to stabilize p53 by phosphorylation.[5,10] However, there is no p53 ortholog present in the yeast genome[11] and in *Drosophila*,

its function appears not to be related with p53 stabilization,[8] suggesting that Bud32/PRPK function is not dependent on p53 in metazoans.

Additionally, Bud32 kinase activity would not be required for its cellular function, because kinase-dead versions of this protein can significantly rescue the Δ*bud32* mutant yeast[12] and our results using analog catalytic mutant versions in *Drosophila* point in the same direction.[8] Another site that has been suggested to be relevant for its activation is an Akt phosphorylation motif R-x-R-x-x-p(S/T) present in the C-terminal region of Bud32/PRPK. This phosphorylation was observed in vitro and in vivo in human cell culture lines and yeast.[6] However, our results have shown that Prpk function does not depend on this putative regulation.[8] Therefore, Bud32/PRPK appears to share an ancient function that does not seem to significantly require its kinase activity or Akt/PKB pathway regulation.

Our results in *Drosophila* showed that Prpk knockdown animals have phenotypes similar to those found in mutants for positive regulators of the PI3K/TOR pathway. This pathway is able to transduce hormonal and nutritional status into animal growth by regulating the protein translation machinery.[8] Here, we present a commentary of our views along with evidence of how this protein complex is related with protein biosynthesis and cell growth.

Bud32/PRPK is Part of the Ancient EKC/KEOPS Complex

Bud32/PRPK has been described as part of an ancient and universally conserved multiprotein complex identified in yeast by two independent laboratories, named KEOPS (kinase, putative endopeptidase and other proteins of small size)[13] and EKC (endopeptidase-like kinase chromatin-associated).[14] KEOPS in yeast comprises four subunits (*Drosophila* orthologs are indicated in parentheses): Pcc1p (CG42498), the ATPase Kae1p (CG4933), the kinase Bud32p (Prpk, CG10673) and Cgi121p, which is not conserved in *Drosophila*. Yeast EKC also includes Gon7p, which appears to be present only in fungal genomes.[14]

KEOPS/EKC complex has been implicated in telomeres maintenance,[13] transcriptional regulation,[14] budding site selection[15] and chemical modification of tRNAs (tRNAs).[16,17] The core constituents of the KEOPS complex are present in *Drosophila*, but their physical interactions have not been reported yet. Whether the KEOPS complex is involved in only one biological pathway or process that has pleiotropic effects, or instead, it is directly involved in several pathways is still unclear. Our recent data concerning the physiological consequences of Prpk depletion in *Drosophila* and the phenotypes observed in yeast when components of the EKC/KEOPS complex are absent allow us to suggest that growth defects observed in KEOPS mutant backgrounds are likely to be explained in terms of a specific pathway affected causing the growth phenotypes.

This complex has been shown to be involved in telomere maintenance.[13] In contrast to yeast and human, *Drosophila* telomere structure is preserved by a mechanism that relies on tandem transposable elements.[18,19] The intrinsically different mechanisms governing this process in these linages discard it as a plausible explanation for the common growth phenotypes observed.

Although the chromatin immunoprecipitation data reported by Kisseleva-Romanova et al., shows that the EKC complex associates with transcribing genes,[14] linking its function with transcriptional regulation, further evidence suggests that the observed growth phenotypes are more likely to be caused by protein synthesis defects. In yeast, it has been shown that KEOPS complex integrity is required for Gcn4p translational repression. Gcn4p as a transcriptional activator triggers the expression of genes responsible for amino acid biosynthesis in response to amino acid shortage. Gcn4p translational de-repression is activated by protein synthesis initiation problems. Accordingly, an enrichment of its targets has been detected in KEOPS mutants,[20] suggesting that the underlying cause of the growth phenotypes is more likely related with translational problems rather than with transcriptional failure. This notion is supported by the synthetic lethality of EKC/KEOPS and eIF5 mutants (eIF5 is required for translation initiation).[20] In agreement with these observations, the defective-growth phenotype observed in *Drosophila* was not rescued by Myc overexpression,[8] a global regulator of genes related with cell growth,[21] indicating that the phenotype observed in flies is unlikely to be associated with generalized problems in the transcription of targets genes associated with cell growth, like ribosomal genes or cyclin D. Moreover, a key constituent of the EKC (Cgi121p) complex is missing in *Drosophila*, implying that EKC members are probably engaged in distinct processes in this organism.

Another hint in this puzzle is the fact that mutants of EKC/KEOPS complex components in yeast have random budding patterns.[15,22] This could also contribute with the EKC/KEOPS mutant slow-growth phenotype. We think this is unlikely to be the underlying cause of the growth defects observed in yeast or *Drosophila*, since we detected a cell shape phenotype only in hemocytes (unpublished results), while growth defects are extended to different linages.[8] Mutants for several ribosomal proteins also display random budding patterns, showing that inefficient translation of particular genes and/or impaired growth rate could result in bipolar budding loss. Consequently, many of the mutants that exhibit strong growth defects are also budding defective (i.e., ribosomal proteins mutants),[22] suggesting that EKC/KEOPS function could be directly related with cell growth control mechanisms, as we have shown for *Drosophila* Prpk.[8]

The EKC/KEOPS Complex is Required for tRNA Modification and Protein Synthesis Control

The presented reasoning, together with our results, point to tRNA modification to be the ancestral function of the KEOPS complex. This function could be linked with the protein synthesis machinery and cell growth, being the highly conserved Kae1 protein the principal responsible of tRNA modification.

tRNAs are substrates for protein synthesis and a central part of the translation machinery in all living organisms. A striking feature of tRNAs are their conserved

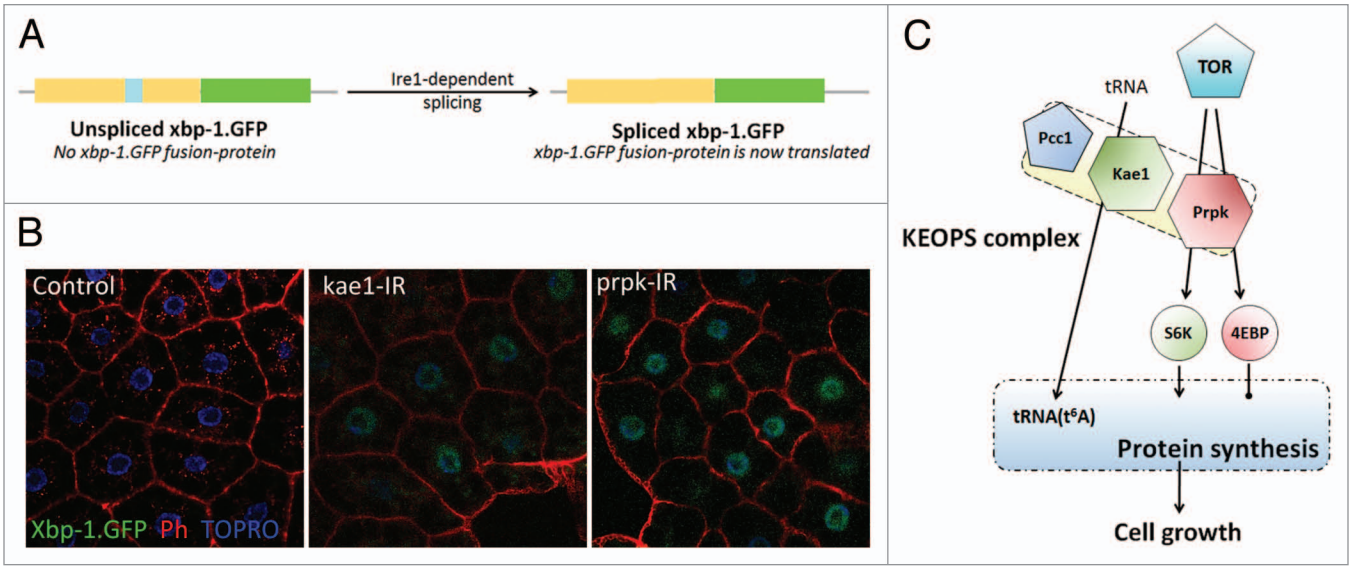

Figure 1. Kae1 or Prpk silencing activates UPR in *Drosophila*. (**A**) UPR activation was detected by using an in vivo xbp1 reporter. This reporter is a fused xbp-1::GFP construct, which upon transcription and UPR induction is spliced by the Ire1 sensor and translated into a Xbp-1::GFP fusion protein. (**B**) Immunofluorescence in *Drosophila* fat bodies against GFP to label xbp1::GFP spliced protein, phalloidin (Ph) to indicate cell boundaries and ToPRO to mark nuclei. In control fat bodies no GFP signal is detected, but when Kae1 or Prpk is depleted GFP signal is detected in the cytoplasm and nuclei of these cells. (**C**) Model representing how KEOPS complex could be operating in protein synthesis and as a consequence, in cell growth. A functional KEOPS complex is able to ensure t^6A-modified tRNAs synthesis through Kae1, and at the same time Prpk is required for TOR kinase activity to promote protein synthesis. In this way, the EKC/KEOPS complex could coordinate the abundance of properly modified substrates and stimulate protein synthesis at the same time. The uncoupling of these functions, observed in mutation or depletion of this proteins, causes a decrease in protein synthesis and, as a consequence, the growth phenotypes observed.

chemical post-transcriptional modifications at specific positions, important in fine-tuning tRNAs structure and function.[23] N6-threonylcarbamoyladenosine (t^6A) is a highly conserved modification occurring at position 37 in tRNAs that pair A-starting codons critical for proper codon recognition and translation accuracy.[20] In *S. cerevisiae* most components of the KEOPS complex (Kae1p, Bud32p and Pcc1p) and the conserved protein Sua5 are required for t^6A synthesis. Mutant yeast for any of these genes presented a slow-growth phenotype.[16,17] Moreover, mutant components of KEOPS that are unable to interact with each other present the same slow-growth phenotype observed in the single null mutants.[12] Furthermore, in *Archeal* genomes the Kae1 and Bud32 coding sequences are fused,[2] supporting the idea that Kae1 and Bud32 depend on each other for their function. Together these observations imply that cells require an intact EKC/KEOPS complex to work properly and if this is not achieved, cells experience growth problems as a result of improper translation. The only ortholog of components of the KEOPS complex in *Escherichia coli*, Kae1 (YgjD) is essential[24] and using an inducible construct it has been shown that YgjD is also required for cell growth.[25] These results suggest that t^6A synthesis is the most primitive function of Kae1, incorporated during evolution into the EKC/KEOPS complex to fulfill physiological requirements of eukaryotic and archeal cells.

Since t^6A modification is required for appropriate translation, a substantial reduction of t^6A-modified tRNAs causes protein synthesis to become error-prone, which in a chronic fashion produces proteins with amino acid substitutions, improper start and stop codon recognition and shifts in reading frames generating aberrant polypeptides. All these aberrations produce anomalous proteins that are more likely to misfold and aggregate and, in the case of proteins trafficking through the secretory pathway, could induce endoplasmic reticulum stress and the unfolded protein response (UPR) as a homeostatic reaction to unfolded or misfolded protein accumulation in this organelle.[26] Considering this, we aimed to detect UPR activation in Prpk and Kae1-knockdown conditions. Using an in vivo reporter (**Fig. 1A**)[27] we observed UPR induction when we knocked down Prpk or Kae1 in the fat body (**Fig. 1B**), suggesting that, as well as in yeast, the components of EKC/KEOPS complex in *Drosophila* are implicated in protein translation accuracy and possibly also in t^6A synthesis. As a part of EKC/KEOPS, Prpk has a structural function but is also necessary for the activity of Kae1. Our results show that Prpk kinase activity is not required for these functions, as overexpression of a kinase dead form does not produce any detectable phenotype.[8] However, and in accordance with its structural role, if Prpk levels are decreased and therefore KEOPS complex conformation disturbed, the unfolded protein response is induced (**Fig. 1B**). Also Prpk is required for TOR activity in *Drosophila*, and probably also in other eukaryotes. This was evidenced by the reduction in S6K and 4EBP phosphorylation, two direct targets of TOR kinase,[8] suggesting that Prpk is required for protein synthesis control through modulating TOR kinase.

The above results suggest that Kae1 and its binding partner in the KEOPS complex, Prpk, are required for protein translation homeostasis: Kae1 produces a key substrate, the t^6A-modified tRNAs and Bud32/Prpk regulates TOR activity; thus both enzymes are essential for proficient protein synthesis. An interesting possibility arising from these observations is that KEOPS complex could act as a permissive element that coordinates the rate of protein synthesis (controlled through S6K and 4EBP phosphorylation by TOR kinase) and the availability of properly modified substrates. In this scenario, if KEOPS complex remains operational the cell has sufficient t^6A-modified tRNAs and adequate TOR activity, allowing to sense the environment conditions and interpret the cell requirements for growth throughout developmental stages (**Fig. 1C**). Consequently the growth deficient phenotype could be explained in terms of inappropriate protein synthesis. When Prpk is silenced we observe a significant decrease in TOR activity and also UPR activation, suggesting that Prpk is also required for correct translation. Similarly, Kae1 knockdown causes UPR activation, consistent with its role in t^6A-modified tRNAs and protein synthesis. Thus knocking down Prpk or Kae1 uncouples their coordinated functions, producing similar phenotypes including UPR activation, increased apoptosis and reduced cell growth.

In conclusion, we visualize KEOPS complex as an ancestral multiprotein complex that has been subjected to the inclusion of different subunits for diverse processes, like telomere dynamics or transcription. However, our results and the examination of previous reports presented here suggest that the core, and perhaps the original function of this complex, is related with translation homeostasis. Accurate protein synthesis is required for cell growth and homeostasis; consequently incorrect protein synthesis leads to an imbalanced cell physiology, which impacts at several levels with pleiotropic effects that are evidenced as a growth deficient phenotype.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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