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DREF is required for cell and organismal growth in *Drosophila* and functions downstream of the nutrition/TOR pathway

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ABSTRACT

Nutrient availability is a key determinant of animal growth. The conserved insulin/PI3 kinase and TOR kinase signaling pathways are two of the best characterized regulators of cell and tissue growth in response to nutritional conditions. Studies in *Drosophila* larvae show that one mechanism by which these pathways drive growth is by regulating the expression of metabolic genes, especially those genes required for protein synthesis. Here we examine a role for the transcription factor DREF in mediating some of these transcriptional and growth responses. We find that loss of DREF leads to a decrease in organismal growth. These effects are in part due to a requirement for DREF function in cell-autonomous growth. We also uncover a non-autonomous role for DREF activity in the larval fat body. Previous studies show that activation of TOR in the fat body couples nutrition to insulin release from the brain; we find that inhibition of DREF in the fat body can phenocopy effects of nutrient deprivation and fat-specific TOR inhibition, leading to a reduction in systemic insulin signaling, delayed larval growth and smaller final size. Using genetic epistasis, we find that DREF is required for growth downstream of TOR, but not insulin/PI3K signaling. Moreover, we show that TOR can control DREF mRNA levels, in part via the transcription factor dMyc. Finally we show that DREF is required for normal expression of many ribosome biogenesis genes, suggesting that one mechanism by which DREF is required for growth is through the control of protein synthetic capacity. Together these findings suggest DREF is an essential transcription factor in the nutritional control of cell and tissue growth during *Drosophila* development. Given that DREF is conserved, this role may also be important in the control of growth in other animals.

Introduction

Multicellular organisms respond to a variety of developmental and environmental cues to control their growth. One important environmental cue is nutrition. Animals often live in conditions where food abundance varies. Thus as they develop, animals must coordinate their cellular metabolism with changes in nutrient availability in order to support tissue and body growth. Defects in this coordination can impair development and lead to growth disorders and lethality.

Studies in *Drosophila* larvae have been pivotal in identifying mechanisms that couple nutrition to tissue and body growth during animal development. Upon larval hatching, the intake of dietary amino acids is required to initiate and maintain cell growth and cell cycling in almost all tissues (Britton and Edgar, 1998). In nutrient rich conditions, larvae continue to develop and increase in mass approximately 200-fold over four days (Robertson, 1966). In contrast, starvation for dietary amino acids blocks larval cell growth and cell cycling, particularly in the polyploid cells that make up the bulk of the larval tissues, such as fat, muscle, gut, salivary gland and epidermis (Britton and Edgar, 1998). An overwhelming body of work points to two conserved signaling pathways that link dietary amino acids to cell and tissue growth.

i. The first is the insulin/insulin-like growth factor signaling pathway. *Drosophila* contains seven insulin-like peptides (dILPs) (Brogiolo et al., 2001). In response to dietary amino acids, three of these dILPs (dILPs 2, 3 and 5) are expressed and released from neurosecretory cells in the brain (Geminard et al., 2009; Ikeya et al., 2002). These dILPs then circulate in the larval haemolymoph and trigger growth in target cells by binding to the insulin receptor and activating a conserved PI3 kinase/Akt kinase signaling pathway (Grewal, 2009; Teleman, 2010). In this way, the insulin pathway represents a primary endocrine response regulated by dietary nutrients.
ii. The second is the Target-of-Rapamycin (TOR) kinase pathway (Foster and Fingar, 2010; Inoki and Guan, 2006; Wullschleger et al., 2006). TOR exists in two different protein complexes

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(TORC1 and TORC2) with TORC1 being the predominant growth regulator. In all eukaryotes, extracellular nutrients and amino acids can cell autonomously activate intracellular signaling cascades that lead to increased TORC1 activity (Dann and Thomas, 2006; Sengupta et al., 2010; Wang and Proud, 2009). A large body of work, predominantly in mammalian cell culture, has also suggested that the insulin/PI3K pathway can be coupled to activation of TOR (Dann and Thomas, 2006; Foster and Fingar, 2010; Inoki and Guan, 2006; Sengupta et al., 2010; Wang and Proud, 2009). However, genetic studies suggest that the proposed signaling mechanisms underlying this coupling may be dispensable for tissue growth in vivo (Dong and Pan, 2004; Pallares-Cartes et al., 2012; Schleich and Teleman, 2009). In *Drosophila*, as in all eukaryotic cells studied, activation of TOR can promote cell-autonomous increases in growth (Oldham, 2000; Zhang et al., 2000). In addition, recent studies have identified non-autonomous roles for TOR signaling in the control of overall body growth. A pertinent example for our work here is the role of TOR activation in the larval fat body, which, in response to dietary nutrients, leads to the relay of a fat-to-brain signal that promotes dILP release and systemic insulin signaling (Colombani et al., 2003; Geminard et al., 2009).

The roles of insulin/PI3K and TOR signaling as regulators of growth are highly conserved in metazoans. In particular, the networks of intracellular signaling molecules that both propagate insulin receptor signaling and that link extracellular nutrients to TOR activation are very similar among all animals. What is less clear is the identity of the key effectors that couple these pathways to changes in cellular metabolism essential for growth.

One mechanism by which insulin and TOR signaling pathways may influence cellular metabolism is through changes in gene expression. Several microarray studies in *Drosophila* larvae have shown that dietary nutrients can alter transcript levels of a large number of genes, particularly those involved in cellular metabolism (Li et al., 2010; Teleman et al., 2008; Zinke et al., 2002). The role of two conserved transcription factors, FOXO and dMyc, have been best studied in this regard. The insulin/PI3K pathway inhibits FOXO by phosphorylating it and relocating it to the cytoplasm (Hay, 2011). Conversely, upon starvation (and hence reduced insulin/PI3K activity), FOXO is dephosphorylated and can enter the nucleus where it binds to gene promoters to regulate transcription (Hay, 2011). Indeed, many of the changes in metabolic gene expression induced by starvation are reversed in *foxo* mutant larvae, suggesting a prominent role for FOXO in mediating the effects of dietary restriction (Teleman et al., 2008). dMyc is another important mediator of nutritional gene expression, particularly the transcription of genes involved in ribosome biogenesis (Grewal et al., 2005; Li et al., 2010; Teleman et al., 2008). Both the insulin/PI3K and TOR branches of the nutrient-sensing pathway can regulate Myc, at the mRNA and protein levels respectively (Parisi et al., 2011; Teleman et al., 2008). Further transcription factors known to regulate gene expression downstream of TOR include SREBP, involved in lipid and sterol biosynthesis, and Sugarbabe, which mediates the effect of nutrient deprivation on dILP release (Porstmann et al., 2008; Varghese et al., 2010).

In this paper, we explore the role of the transcription factor DREF (DNA Replication Related Element Binding Factor) as an additional mediator of nutrition-dependent growth and metabolic gene expression in *Drosophila*. DREF is a 709 amino acid polypeptide that acts as a homodimer to bind to 5′-TATCGATA-3′ sites (DRE motif) in gene promoters (Hirose et al., 1993). It has been best studied for its role in cell cycle regulation and in the transcriptional activation of a variety of genes involved in DNA replication and cell proliferation (Matsukage et al., 2008). However, our investigation here was prompted by two findings: first, we found that the DRE motif is enriched in the promoters of many ribosome biogenesis genes whose expression is regulated by nutrition. Second, DREF can function as part of a multi-protein transcriptional complex that includes the TBP-related factor TRF2 (Hochheimer et al., 2002); a previous paper showed by both ChIP and transcript analysis that TRF2 was associated with, and required for, expression of many ribosomal protein genes in *Drosophila* (Isogai et al., 2007). These findings raised the possibility that, in addition to the control of cell cycle genes, DREF may be required for cellular metabolism and hence cell growth. Our findings here support this model: we show that DREF is required for both cell-autonomous and non-autonomous cellular and organismal growth, and interestingly, DREF appears to be required downstream of the TOR pathway while acting independently from the insulin/PI3K pathway.

Materials and methods

Fly stocks

All stocks and crosses were raised at 25 °C and maintained on standard *Drosophila* media (150 g agar, 1500 g cornmeal, 315 g yeast, 675 g sucrose, 1875 g D-glucose, 240 ml propionic acid per 34.5 L water). The following fly stocks were used: w^{1118}; *yw*; $dref^{KG09294}$/*CyO*; $dref^{NP4719}$/*CyO*; $dref^{KG09294}$, *ubi-GFP*, *FRT40A*/*CyO*; *ubi-GFP*, *FRT40A*/*CyO*; $pten^{djc89}$, *ubi-GFP*, *FRT40A*/*CyO* (Gao et al., 2000); $tor^{\Delta P}$, *ubi-GFP*, *FRT40A*/*CyO*; *RpS13*, *ubi-GFP*, *FRT40A*/*Sm6-tm6B*; *FRT82B*/*tm6B*; *FRT82B*, $rheb^{26.2}$/*tm6B*; *act UAS-dref RNAi* (*weaker RNAi on III*) (Hyun et al., 2005); *UAS-dref RNAi* (*stronger RNAi on X*) (Hyun et al., 2005); *UAS-dMyc*; $tor^{\Delta P}$/*CyO* (Zhang et al., 2000); act > *cd2* > *gal4*, *UAS GFP*; *da-gal4*; *mef-gal4*; *cg-gal4*; *r4-gal4*; *ey-gal4*.

Egg collection

Adult flies were allowed to lay eggs on grape juice agar plates supplemented with yeast paste for 4 h at 25 °C. 24 h after egg laying (AEL) the plates were cleared of larvae. All larvae which hatched in the next 4 h were placed in food vials in groups of 50 and allowed to develop.

Microscopy

All immunofluorescence images were obtained on a Zeiss Observer Z1 microscope using Axiovision software. Larval size, pupal volume and adult eye and wing imaging were obtained using a Zeiss Stereo Discovery V8 microscope using Axiovision software. Microscopy and image capture was performed at room temperature and captured images were processed using Photoshop CS5 (Adobe).

Mitotic recombination, clone and cell size analysis

Mosaic *dref RNAi* cell clones in the fat body were induced using the heat-shock *flp-out* method (Britton et al., 2002). Tissues were dissected at 120 h AEL, fixed in 8% paraformaldehyde, stained with Hoeschst (Invitrogen) and TRITC-conjugated phalloidin (Invitrogen) and mounted in Vectashield (Vector Laboratories Inc.). Mitotic recombination was performed using the *flp*/FRT method (Xu and Rubin, 1993). For the fat body cell analysis, we performed a 6 h egg collection followed by a 1 h heat-shock at 37 °C. Larvae were transferred to food vials 24 h after heat-shock. Larvae were inverted and fixed in 8% paraformaldehyde at 120 h AEL and tissues were stained with Hoescht and TRITC-conjugated