**Research Article**

**Drosophila TRF2 and TAF9 regulate lipid droplet size and phospholipid fatty acid composition**

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**Abstract**

The general transcription factor TBP (TATA-box binding protein) and its associated factors (TAFs) together form the TFIIID complex, which directs transcription initiation. Through RNAi and mutant analysis, we identified a specific TBP family protein, TRF2, and a set of TAFs that regulate lipid droplet (LD) size in the *Drosophila* larval fat body. Among the three *Drosophila* TBP genes, *trf2*, *tbp* and *trf1*, only loss of function of *trf2* results in increased LD size. Moreover, TRF2 and TAF9 regulate fatty acid composition of several classes of phospholipids. Through RNA profiling, we found that TRF2 and TAF9 affect the transcription of a common set of genes, including peroxisomal fatty acid β-oxidation-related genes that affect phospholipid fatty acid composition. We also found that knockdown of several TRF2 and TAF9 target genes results in large LDs, a phenotype which is similar to that of *trf2* mutants. Together, these findings provide new insights into the specific role of the general transcription machinery in lipid homeostasis.

**Author summary**

Lipid droplets (LD) are main lipid storage structures in most cells. The size of LDs varies greatly in different cell types or different metabolic states to accommodate cellular functions and metabolism demands. How cells regulate the lipid storage and LD dynamics is not fully understood. Here, we identified that general transcription factors, including a specific TBP (TATA-box binding protein) family protein TRF2 (TBP-related factor 2) and several TAFs (TBP-associated factors), regulate LD size in the fruitfly larval fat body. Moreover, quantitated lipid analysis reveals that TRF2 and TAF9 affect the fatty acid composition of several classes of phospholipids. We showed that TRF2 and TAF9 regulate transcription of several target genes, including peroxisomal fatty acid β-oxidation-related genes which likely mediate the effect of TRF2 and TAF9 on phospholipid fatty acid composition. We also found that overexpression of some target genes restores the LD
phenotype in trf2 mutants. Our findings therefore reveal specific roles of general transcription factors in lipid homeostasis.

Introduction

The Pol II (RNA polymerase II)-GTF (general transcription factor)-Mediator-TF (transcription factor)-Effector (target genes) axis of eukaryotic transcriptional regulation has been well established in many biological processes. Biochemical, cellular and physiological studies have discovered that adipocyte differentiation and lipid homeostasis in adipose tissue are regulated by an adipogenic transcription cascade including C/EBPs and PPARs, a lipogenic enzymatic cascade and an increasing list of specific transcription factors such as SREBP, LXR, FXR, NHR-49 and HNF4α [1–3]. Previous studies also revealed that by interacting with specific transcription factors, general transcription machineries, including the Mediator subunits MED1, MED13, MED14, MED15, MED23 and MED25, play important roles in lipid metabolism [4–10]. The Mediator complex bridges general transcription factors and Pol II to specific transcription factors to regulate transcription. However, it is not fully understood whether general transcription factors exhibit specificities in regulating lipid metabolism.

Adipocytes store neutral lipids in lipid droplets (LDs), which are intracellular organelles consisted of a monolayer of phospholipids, a neutral lipid core, and associated proteins [11]. The size of LDs varies greatly in different cell types to accommodate distinct cellular functions. White adipocyte usually contains a large unilocular LD for lipid storage, while brown adipocyte has many small LDs for rapid lipolysis. It is well known that the content of the lipid core, the composition of monolayer phospholipids and the protein machinery for LD fusion affect the size of LDs [12–16]. In addition, taking advantage of genetic and cell based-RNAi screens, systematic studies in C. elegans and Drosophila cultured cells by lipid staining and/or imaging, and in Drosophila adults by measuring total levels of triacylglycerol (TAG) have identified numerous genes and cellular pathways involved in the regulation of lipid storage and LD dynamics [17–21]. Along with other functional studies [22–26], these findings provide valuable clues as to the complicated regulation of lipid storage and LD dynamics. However, our understanding of the lipid storage network and regulation of LD dynamics is far from clear.

In this study, we identified that several components of the general transcription factor TFIID complex, including a specific TBP (TATA-box binding protein) family protein TRF2 (TBP-related factor 2) and several TAFs (TBP-associated factors), regulate LD size in the Drosophila larval fat body, which is the adipose tissue in flies. Unlike TBP, which binds to TATA-containing promoters and initiates Pol II-dependent gene transcription, TRF2 acts as a core promoter-selective factor and regulates transcription from TATA-less promoters [27–30]. In Drosophila, trf2 is required for several specific biological processes such as embryonic development, germ cell differentiation and metamorphosis [31, 32].

Drosophila has three TBP genes and only loss of function of trf2 results in large LD phenotype. Moreover, lipidomic analysis reveals that TRF2 and TAF9 also affect the fatty acid composition of several classes of phospholipids. We showed that TRF2 and several core TAFs affect transcription of several target genes related to lipid metabolism. The regulatory effect of TRF2 and TAF9 on phospholipid fatty acid composition is most likely mediated by genes involved in the peroxisomal fatty acid β-oxidation. We also found that overexpression of some target genes restores the LD phenotype in trf2 mutants. Therefore, our study reveals specific roles of general transcription factors, namely TRF2 and TAF9, in lipid homeostasis.
Results

TAF9 affects *Drosophila* fat body LD size

To systematically identify genes that regulate lipid storage in adipose and non-adipose tissues, we used the tissue-specific *Gal4-UAS* system to perform an RNAi screen in *Drosophila* larval fat body and salivary gland with *ppl-Gal4* [33]. We found that knockdown of the general transcription factor TFIID complex component TAF9 results in enlarged LDs in the fat body (Fig 1A). The diameter of LDs stained with BODIPY dye was measured and quantified. In *ppl-Gal4* controls, the average size of LDs is around 9 μm and the largest is around 14 μm. In *taf9* RNAi fat body, the average size of LDs increases to around 11 μm and the largest is over 20 μm (Fig 1B).

To confirm the results from RNAi, we examined the *taf9* mutant phenotype. *taf9* is also known as *enhancer of yellow 1*, *e(y)1*, and was originally reported to affect the Yellow phenotype [34]. In another study, *taf9* partial loss-of-function mutants were found to impair female fertility and oogenesis [35]. Furthermore, *taf9* was reported to be involved in the transcriptional regulation of Notch signaling [36]. We generated two N-terminal deletion mutants of *taf9* through P-element imprecise excision (Fig 1C). Both mutants are homozygous lethal after the wandering 3rd instar larval stage. A few of mutant larvae can form white prepupa and then die. We therefore examined the LD phenotype in the fat body of active wandering 3rd instar mutant larvae. In *taf9*17 fat bodies, the average size of LDs increases to around 12 μm and the largest one is over 30 μm. In *taf9*20 fat bodies, the average size of LDs is around 15 μm and the largest is over 34 μm (Fig 1D and 1E). To more specifically describe the large LD phenotype, we quantified the size of three largest LDs in individual fat cells. In wild type, the average size of the largest LDs is around 11 μm, while in *taf9*17 and *taf9*20 mutants, it increases to around 19 μm (Fig 1F). These results are consistent with the *taf9* RNAi results. We also examined the phenotype of *taf9*17 mutants at both 2nd and early 3rd instar larval stages. There was no significant difference in LD size between *taf9*17 mutants and controls (S1 Fig).

To further validate the mutant phenotype, we next performed rescue experiments. The large LD phenotype of *taf9*17 and *taf9*20 mutants was rescued by overexpressing *taf9* in the fat body using *ppl-Gal4*, indicating that *taf9* functions autonomously in fat body to regulate LD size (Fig 1D and 1E). Together, the phenotypic analysis of RNAi knockdown animals and P-element-derived knockout mutants indicate that *taf9* plays an important role in LD size regulation.

TFIID core complex genes are required for LD size regulation

TAF9 is one subunit of TFIID, a multi-subunit complex composed of TBP and TAFs. TAF4, TAF5, TAF6, TAF9 and TAF12 form a functional core subcomplex within TFIID [37]. To investigate the involvement of the other TFIID core TAFs in LD size regulation, we knocked them down by crossing available RNAi lines to *ppl-Gal4*. The fat body expression of these taf genes and knockdown efficiency were examined by qRT-PCR. As expected, the expression levels of these genes decreased significantly in the fat body with RNAi (Fig 2C). Phenotypically, RNAi of *taf4*, *taf5*, *taf6* and *taf12* all result in enlarged LDs in the fat body (Fig 2A and 2B and S1 Table). For example, in *taf4* RNAi fat body, the average size of LDs increases to around 13 μm and the largest is over 20 μm. These results suggest a general requirement for the TFIID core complex in LD size regulation. Moreover, knockdown of *taf1*, which is a non-core complex component, also leads to a large LD phenotype (Fig 2A and 2B). Knockdown of other non-core complex components did not affect LD size (S1 Table). It is possible that either they are not required in this context or the RNAi efficiencies were not high enough to cause a measurable LD phenotype.
Among the three TBP family proteins, TRF2 is specifically involved in LD size regulation.

How do the aforementioned TAF proteins affect LD size? TAF proteins associate with TBP to form the TFIID complex, which binds to the core promoter and initiates assembly of the PIC (preinitiation complex) to facilitate Pol II-dependent transcription initiation. There are three TBP homologs in Drosophila: the founding member TBP, a closely related member TRF1, and a distantly related member TRF2 [38, 39]. TBP and TRF2 exist in all metazoans; TRF1 is specific to insects, while vertebrates have TRF3. TBP binds to the TATA box, while TRF3 was reported to recognize TATA-less promoters. We firstly knocked down all three TBP family members (tbp, trf1 and trf2) in the fat body by RNAi. Only knockdown of trf2 results in increased LD size: the average size of LDs increases to around 11 μm and the largest is over 22 μm (Fig 3A and 3B). This phenotype is reminiscent of that generated by knockdown of taf genes. Since the expressions of all three genes in the fat body are reduced by RNAi (Fig 3C), these data suggest that the TFIID core complex mediates TRF2-dependent transcription to regulate LD size. To further investigate whether trf2 also regulates LD size in adult stage, we...
knocked down trf2 in adult fat body with cg-Gal4. Knockdown of trf2 leads to more small LDs compared to controls (S2 Fig), while knockdown of taf9 causes lethality at pupal stage. These results indicate that TRF2 plays an important role to regulate LD size in both larval and adult stages.

To further verify our findings with trf2 RNAi, we next investigated the fat body phenotype of tbp, trf1 and trf2 mutants. tbp^{f00190} is a piggyBac insertion mutation while trf2^{G0071} has a P-element insertion mutation in the intron and displays lethality during metamorphosis, allowing us to investigate the fat body phenotype in wandering 3rd instar larvae. Similar to the RNAi results, the fat cells in trf2^{G0071} mutants also have large LDs: the average size is around 15 μm and the largest is over 30 μm (Fig 3E and 3F). When the three largest LDs in each fat cell are quantified, the average size of LDs in trf2^{G0071} mutants is around 19 μm (Fig 3G). These results indicate that TRF2 is specifically involved in LD size regulation. Moreover, trf2^{G0071} mutants have enlarged LDs in the fat body at both 2nd and early 3rd instar larval stages, indicating that TRF2 is also required for LD size regulation at early developmental stages (S1 Fig).

Drosophila trf2 encodes two protein isoforms: a 632-amino-acid short isoform (TRF2s) containing the DNA binding domain, which shows similarity to the core domain of TBP; and a 1715-amino-acid long isoform (TRF2l) in which the identical short TRF2 sequence is
TRF2 and TAF9 regulate lipid droplet size

preceded by a long N-terminal region [31]. To examine the contribution of different TRF2 isoforms to the LD phenotype observed in trf2<sup>G0071</sup> mutants, we performed rescue experiments by expressing the individual isoforms. We found that the large LD phenotype of trf2<sup>G0071</sup> mutants was rescued by expressing either TRF2 isoform in the fat body, indicating that the large LD phenotype is due to trf2 deficiency and trf2 plays an autonomous role in regulating LD size (Fig 3E and 3F). Furthermore, the rescue data reveal that the long N-terminal region of TRF2<sub>l</sub> is not required for the function of TRF2 in LD size regulation. In addition, overexpression of trf<sub>2s</sub> with ppl-Gal4 at 29°C leads to small LDs. The average size of LDs is around 8 μm and the largest is around 13 μm (Fig 3H and 3I). Overexpression of trf2<sub>l</sub> with ppl-Gal4

Fig 3. Among three TBP family members in Drosophila, only TRF2 specifically regulates LD size. (A) Knockdown of trf2 in larval fat body leads to increased LD size whereas knockdown of tbp or trf1 has no significant phenotype. Scale bar represents 50 μm. (B) Quantification of LD diameter in (A). Data were analyzed by one-way ANOVA with Dunnett’s multiple comparisons test. Error bars represent ±SD. ***: p < 0.001; n.s.: nonsignificant. (C) Relative mRNA levels of tbp, trf1 and trf2 in fat bodies from different backgrounds. Error bars represent ±SD. (D) Analysis of tbp and trf1 mutant fat cell clones. Mutant fat cells (GFP-negative) are marked by white dashed lines and twin-spot control cells (GFP-positive) are outlined by yellow dashed lines. LDs were stained by LipidTOX (red) and nuclei were stained by DAPI (blue). There is no difference between tbp or trf1 mutant fat cells and control fat cells. Scale bar represents 50 μm. (E) BODIPY staining of LDs in 3rd instar larval fat bodies of different genetic backgrounds. trf2<sup>G0071</sup> mutants have an enlarged LD phenotype, which is fully rescued by expression of either trf2<sub>l</sub> or trf2<sub>s</sub> in the fat body. Scale bar represents 50 μm. (F) Quantification of LD diameter in (E). Data were analyzed by one-way ANOVA with Tukey’s multiple comparisons test. Error bars represent ±SD. ***: p < 0.001. (G) Quantification of the diameter of the three largest LDs per cell of w<sup>1118</sup> and trf2<sup>G0071</sup> mutants. Error bars represent ±SD. ***: p < 0.001. (H) Overexpression of trf2<sub>s</sub> results in small LDs in larval fat body. Scale bar represents 50 μm. (I) Quantification of LD diameter in (H). Error bars represent ±SD. ***: p < 0.001.

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at 29˚C leads to developmental arrest at early 3rd instar larval stage. Together, our findings derived from phenotypic analysis of RNAi knockdown and mutant animals reveal a specific role of TRF2, but not TBP or TRF1, in LD size control.

TRF2 and TAF9 regulate the fatty acid composition of phospholipids

Previous studies have revealed that the content of neutral lipid core and/or the levels of monolayer phospholipids contribute to LD size. To reveal the effects of TRF2 and TAF9 on lipid composition, we profiled the level of phospholipids and neutral lipids in the larval fat bodies of different backgrounds. Assays were done in triplicate. Data were analyzed by one-way ANOVA with Dunnett’s multiple comparisons test. Error bars represent ±SEM. ***: p < 0.001; **: p < 0.01; *: p < 0.05. Ergo, ergosterol; Free Cho, free cholesterol; DAG, diacylglycerol; TAG, triacylglycerol; CL, cardiolipin; LPA, lysophosphatidic acid; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine. (B-H) The relative level (normalized to total phospholipids) of species with different fatty acid chain lengths in PA (B), PC (C), PG (D), PI (E), PS (F), PE (G), and TAG (H) in the larval fat bodies of different backgrounds. Error bars represent ±SEM.

We also compared the fatty acid composition of major phospholipids and TAG (Fig 4B–4H). We noticed changes in the phospholipid fatty acid chain length in trf2 and taf9 RNAi. Several classes of phospholipids in trf2 and taf9 RNAi tend to be composed of fatty acids with long chain lengths. When we compared the relative amounts of major phospholipids based on the total fatty acid chain length, there are more phospholipid species with longer fatty acid chains in trf2 and taf9 RNAi fat bodies compared with ppl-Gal4 controls. This trend was...
obvious in phosphatidic acid (PA), PC, PG and PI but less significant in phosphatidylethanolamine (PE), PS and TAG (Fig 4B–4H). These results suggest that TRF2 and TAFs may regulate the expression of a specific set of genes which are important for controlling LD size and phospholipid fatty acid composition.

Identification of trf2 and taf9 target genes

To identify the potential target genes regulated by TRF2 and TAF9 in controlling LD size and phospholipid fatty acid composition, we performed RNA-seq to profile global gene expression patterns of the fat body in controls, trf2 RNAi, trf2G0071 mutants and taf917 mutants. We defined differentially expressed genes as showing more than twofold difference in gene expression and having a FDR (False Discovery Rate) cutoff of ≤ 0.001 (Fig 5A). Compared to the ppl-Gal4 control, there are 2574 down-regulated genes and 1237 up-regulated genes in trf2 RNAi (Fig 5B). In trf2G0071 mutants, 4550 genes are down-regulated and 871 genes are up-regulated compared to w1118 controls (Fig 5B). In both trf2 RNAi and trf2G0071 mutant fat bodies, there are many more down-regulated genes than up-regulated genes, consistent with the positive role of trf2 in gene transcription. The up-regulated gene expression in trf2 RNAi or trf2G0071 mutants may be due to secondary effects. The fact that there are more down-regulated genes in trf2G0071 mutants than trf2 RNAi may be caused by the background difference between trf2G0071 mutants and w1118 controls or differences in the strength of the loss of function between RNAi and mutants. Importantly, there is a significant overlap (2186/2574, 85%) of down-regulated genes between trf2 RNAi and trf2G0071 mutants, indicating that the results are valid. For taf917 mutants, we found 766 down-regulated genes and 493 up-regulated genes compared to w1118 controls (Fig 5B). Compared to gene expression changes in trf2G0071 mutants or trf2 RNAi, we found fewer differentially expressed genes in taf917 mutants, suggesting that taf9 may only participate in some trf2-regulated transcription events. In addition, partial overlap (397/766, 52%) of down-regulated genes between taf917 mutants and trf2G0071 mutants suggests that TAF9 also participates in transcription regulation mediated by other TBP proteins (Fig 5B).

Inactivation of either trf2 or taf9 causes similar LD phenotype, suggesting that they may have common target genes. Therefore, we next focused on the down-regulated genes that overlap between trf2 RNAi, trf2G0071 and taf917 mutants. 181 genes are down-regulated in all three groups, while 46 genes are only down-regulated in taf917 mutants and trf2 RNAi, and 216 genes are only down-regulated in trf2G0071 mutants and taf917 mutants (Fig 5B). Gene ontology (GO) term analysis of the 181 genes shared by all groups showed enrichment of several categories of biological process, including metabolic processes involving various metabolites such as fatty acids, carbohydrates and aminoglycans. This suggests that trf2 and taf9 may play important roles in metabolic regulation in the fat body. Furthermore, there are also other significant GO term categories such as transport, protein alkylation and developmental processes (Fig 5C). We did not find obvious candidate genes that have been reported to cause large LD phenotypes, such as CCT or Plin1, suggesting that trf2 and taf9 may not directly regulate these genes.

We next investigated the regulatory sequence elements in the promoters of the genes that are regulated by TRF2 and TAF9. We performed an unbiased analysis of the promoters of the 181 genes to identify motifs associated with known TFs or de novo motifs. Core promoters (-100 to +50 relative to +1 of the transcription start site) and extended promoters (-500 to +50) were analyzed by HOMER program. A Trithorax-like (TRL)-binding motif and a new de novo motif1 are highly enriched in core promoters, while Twist (TWI)-binding motif and DREF (DRE-binding factor) motif are highly enriched in extended promoters (Fig 5D). These results
suggest that TRF2 and TAF9 may cooperate with these transcription factors to regulate transcription of target genes. The significant enrichment of DREF binding motif in the extended promoters is consistent with previous studies showing that DREF exists in a multisubunit TRF2-containing complex [27] and that DRE (DNA replication-related element) is strongly associated with TRF2-bound promoters [28].
TRF2 and TAF9 acts on genes related to peroxisomal fatty acid β-oxidation to regulate the fatty acid composition of phospholipids

The GO term analysis of 181 potential TRF2 and TAF9 target genes identified two genes, CG4586 and CG9527, belong to the GO term category of fatty acid catabolism. CG4586 and CG9527 encode ACOX (acyl-CoA oxidase) which is involved in peroxisomal fatty acid β-oxidation by converting acyl-CoA to trans-Δ²-enoyl-CoA [41]. In addition, two genes that are down-regulated in trf2 RNAi and taf917 mutants, CG9149 and CG9577, encode β-ketoacyl-CoA thiolase and ECH (enoyl-CoA hydratase) respectively. Both of these enzymes are involved in peroxisomal fatty acid β-oxidation. Peroxisome β-oxidation generates medium-chain (C≤14) fatty acid-CoA by the catabolism of very long-chain (C≥22) fatty acids (VLCFAs) and some long-chain (C16-C20) fatty acids (LCFAs) in mammals [42]. In C. elegans, defects in the peroxisomal fatty acid β-oxidation pathway cause LD expansion associated with altered fatty acid composition in both total lipids and TAG and the supersized LD phenotype, reminiscent of the phenotype of trf2Δ and taf917 mutants [43]. We found that knockdown of either CG4586 or CG9527 leads to significantly increased LD size, although it is not as strong as in trf2Δ or taf917 mutants (Fig 6A and 6B). The LD phenotype was also confirmed with independent RNAi lines (S2 Table). Therefore, we further explored the function of CG4586 and CG9527, and the connection between trf2/taf9 and CG4586/CG9527.

There are six ACOX genes (CG4586, CG5009, CG9527, CG9707, CG9709 and CG17544) in the Drosophila genome, among which only CG4586 and CG9527 have significantly enriched gene expression (4.9 fold and 3.2 fold respectively) in the larval fat body (FlyAtlas). Transcription of both CG4586 and CG9527 was dramatically reduced in the fat body of trf2Δ and taf917 mutants. In contrast, transcription of the other four ACOX genes was largely unaffected by trf2 and taf9 mutations based on our RNA-seq data.

Since ACOX functions in the homeostasis of VLCFAs, LCFAs and medium chain fatty acids, we further investigated the effects of CG4586 and CG9527 on lipid composition. We profiled the levels of phospholipids and neutral lipids in the fat body of CG4586/CG9527 double RNAi through lipidomic analysis (Fig 6C). The levels of most phospholipids and neutral lipids, when normalized to total phospholipids, do not change remarkably compared to the control. Only cardiolipin (CL) is significantly increased in CG4586/CG9527 double RNAi. Similar to the results in trf2 and taf9 RNAi, there are also changes in the phospholipid fatty acid composition in CG4586/CG9527 double RNAi. In PA, PC, PG and PS, the relative amounts of phospholipid species with longer fatty acid chains are increased in CG4586/CG9527 double RNAi fat bodies compared with ppl-Gal4 controls (Fig 6D–6G). Together with the phospholipid profiling of trf2 RNAi and taf9 RNAi (Fig 4B–4G), these results suggest that TRF2 and TAF9 regulate the fatty acid composition of phospholipids likely by modulating the expression of peroxisomal fatty acid β-oxidation genes, such as CG4586 and CG9527.

Next we explored the contribution of these two ACOX genes to the large LD phenotype in trf2Δ mutant fat body. We performed the rescue experiments but found that overexpression of either these two genes in trf2Δ mutant fat body only marginally rescued the large LD phenotype (Fig 6H and 6I). It is possible that the large LD phenotype in trf2Δ mutants is not due to downregulated expression of these two ACOX genes. Alternatively, it is possible that the large LD phenotype is due to impairment of the whole peroxisomal fatty acid β-oxidation pathway, which cannot be restored by simply overexpressing one ACOX gene.

trf2 and taf9 target genes are important for LD size regulation

We next searched for other genes that may mediate the regulation of trf2 on LD size among the 181 genes down-regulated in all three groups. We used available RNAi strains to analyze
the knockdown phenotypes of the genes in larval fat body to explore their contribution to the large LD phenotype. The RNAi results showed that out of 141 genes tested, knockdown of 14 genes lead to an enlarged LD phenotype in fat bodies (Fig 7A and S3 Table). Gene expression analysis by qRT-PCR confirmed that all of these genes have decreased mRNA levels in trf2G0071 and taf917 mutants (Fig 7B). These 14 genes have various molecular functions while some of them or their mammalian homologs have been linked to lipid metabolism based on previous studies (S4 Table). For example, CG5554 and CG9432 encode protein disulfide...
Fig 7. TRF2 affects LD size by directing transcription of several target genes. (A) Quantification of the diameter of the three largest LDs per cell in fat bodies of larvae with different RNAi treatments. RNAi of 14 target genes leads to enlarged LDs. Data were analyzed by one-way ANOVA with Dunnett’s multiple comparisons test. Error bars represent ±SD. ***: p < 0.001; **: p < 0.01; *: p < 0.05. (B) Relative mRNA levels of 14 target genes in fat bodies from trf2^G007/1 and taf9^17 mutants. Error bars represent ±SD. (C)
isomerases that were reported to regulate adiponectin secretion and microsomal triglyceride-transfer protein (MTP) activity [44, 45]. Moreover, these two protein disulfide isomerases were reported to associate with LDs in a previous LD proteome study [46] and knockdown of CG9432 affects LD size and distribution in Drosophila S2 cells [19]. The protein Cyp4d1, encoded by CG3656, was also reported to associate with LDs [47]. We also found some new genes, including CG9497, CG11275 and CG11474, which have no previous link to lipid metabolism, suggesting that they may participate in novel mechanisms in lipid storage regulation.

Among the 14 genes that caused enlarged LDs by RNAi, CG10315, CG15632, CG2617, CG9486 and CG9507 resulted in the most robust LD phenotype when knocked down (Fig 7A). To reveal the contribution of these genes to the large LD phenotype caused by trf2 loss of function, we overexpressed them in trf2 G0071 mutants and examined their rescuing activity. As a positive control, expression of FLAG-tagged TRF2s fully rescues the mutant LD phenotype. We found that the expression of CG10315, CG2617, CG9486 and CG9507 partially rescues the LD phenotype of trf2 G0071 mutants, suggesting that these genes mediate LD size regulation by trf2. Expression of CG15632 has no rescuing effects. Therefore, CG15632 may not be a direct downstream target of trf2 and taf9 in LD size regulation (Fig 7C and 7D).

To investigate whether TRF2 directly regulates the expression of these genes, we performed ChIP assays on larval fat bodies expressing functional FLAG-TRF2s and examined TRF2 occupancy on the core-promoter regions of these genes. As expected, TRF2 occupies the core promoter of RpLP1, which is a reported TRF2 target, but not the TATA-dependent Act87E promoter [29]. We found that TRF2 is strongly recruited to the promoters of CG4586, CG9527, CG10315 and CG15632. TRF2 is also slightly detectable on the promoters of CG2617 and CG9486 (Fig 7E). CG10315 encodes the translation initiation factor eIF2B-δ. Some eIFs, such as eIF4G and eIF-4a, were reported to associate with Drosophila LDs [46]. Furthermore, knockdown of several eIFs, including eIF-1A, eIF-2β, eIF3gα, eIF3-S8 and eIF3-S9, causes large and condensed LDs in Drosophila S2 cells [19]. Taken together, these results indicate that TRF2 regulates transcription of several target genes that may play important roles in LD size regulation.

Discussion

This study reveals a rather specific role of TRF2 and TAFs, which are general transcription factors, in regulating LD size. In addition, TRF2 and TAF9 affect phospholipid fatty acid composition, most likely through ACOX genes which mediate peroxisomal fatty acid β-oxidation (Fig 7F).

By binding to their responsive elements in target genes, specific transcription factors like SREBP, PPARs and NHR49, play important roles in lipid metabolism. It is interesting to find that the general transcription machineries, in this case TRF2 and core TAFs, also exhibit specificity in regulating lipid metabolism. In the Drosophila late 3rd instar larval fat body, defects in trf2 cause increased LD size, whereas mutation of the other two homologous genes, tbp and trf1, have no obvious effects on lipid storage. We also found that inactivation of taf genes
causes a similar phenotype to \textit{trf2} mutation, suggesting that TRF2 may associate with these TAF proteins to direct transcription of specific target genes. Moreover, we found that \textit{trf2} mutants have large LDs at both 2\textsuperscript{nd} and early 3\textsuperscript{rd} instar larval stages, suggesting that general transcription factors are also required at early developmental stages for LD size regulation. Interestingly, \textit{taf9} mutants have no obvious phenotype at these stages. It is possible that TAF9 may act as an accessory factor compared to promoter-binding TRF2. This is consistent with the fact that less genes are affected in \textit{taf9} mutants than \textit{trf2} mutants in our RNA-seq analysis. We also found that knockdown of \textit{trf2} in larval and adult fat body leads to different LD phenotype. This may be due to different lipid storage status or different LD size regulatory mechanisms between larval and adult stages.

Our finding adds to the growing evidence supporting a specific role of general transcription factors in lipid homeostasis. For example, knockdown of RNA Pol II subunits such as RpII140 and RpII33 leads to small and dispersed LDs in \textit{Drosophila} S2 cells \cite{19}. Mutation in DNA polymerase delta (POLD1) leads to lipodystrophy with a progressive loss of subcutaneous fat \cite{48}. Furthermore, TAF8 and TAF7L were reported to be involved in adipocyte differentiation \cite{49–51}. Moreover, previous studies showed that several subunits of the Mediator complex interact with specific transcription factors and play important roles in lipid metabolism \cite{4–10}. Added together, these lines of evidence strongly support essential and specific roles of the core/basal transcriptional machinery components in lipid metabolism.

Using RNA-seq analysis, rescue experiments and ChIP-qPCR, we identified several target genes regulated by TRF2 and TAF9. It is possible that other genes may regulate LD size but were missed in our RNA-seq analysis and RNAi screening assay because of either insufficient alterations in genes expression (lower than the twofold threshold) or low efficiency of RNAi. Among all the verified target genes of TRF2 and TAF9, \textit{CG10315}, which strongly rescues the \textit{trf2} mutant phenotype when overexpressed and encodes the eukaryotic translation initiation factor eIF2B-δ, may be a good candidate for further study. Although they are best known for their molecular functions in mRNA translation regulation, eIFs have been implicated in several other processes, including cancer and metabolism. For example, in yeast, eIF2B physically interacts with the VLCFA synthesis enzyme YBR159W \cite{52}. In adipocytes, elf2\textalpha\ activity is correlated with the anti-lipolytic and adipogenesis inhibitory effects of the AMPK activator AICAR \cite{53}. In addition, given the evidence that some eIFs, such as eIF4G and eIF-4a, localize on LDs \cite{46} and knockdown of some eIFs, including eIF-1A, eIF-2β, eIF3ga, eIF3-S8 and eIF3-S9, results in large LDs in \textit{Drosophila} S2 cells \cite{19}, it is important to further explore the specific mechanisms of these eIFs in LD size regulation.

Although TRF2 exists widely in metazoans and shares sequence homology in its core domain with TBP, it recognizes sequence elements distinct from the TATA-box. A previous study has investigated TRF2- and TBP-bound promoters throughout the \textit{Drosophila} genome in S2 cells and revealed that some sequence elements, such as DRE, are strongly associated with TRF2 occupancy while the TATA-box is strongly associated with TBP occupancy \cite{28}. In our study, we also identified that DRE is significantly enriched (p-value<1e-40) in extended promoters of the 181 target genes. We further explored the distribution of TATA-boxes in the core promoters of the 181 target genes compared with all genes and found that the TATA-box is not enriched in the core promoters of TRF2 target genes. The proportion of TATA-box is 0.155 (75 of 484 isoforms) for the 181 target genes while the proportion is 0.217 (7849 of 36099 isoforms) for all genes as the background. These results suggest that TRF2 and TAF9 may regulate the expression of a subset of genes by recognizing specific sequence elements such as DRE but not the TATA-box.

We showed that expression of peroxisomal fatty acid β-oxidation pathway genes, including two acyl-CoA oxidase (ACOX) genes, \textit{CG4586} and \textit{CG9527}, the β-ketoacyl-CoA thiolase gene...
CG9149, and the enoyl-CoA hydratase gene CG9577, is regulated by TRF2 and TAF9. Lipidomic analysis indicates that in the fat body of trf2 and taf9 RNAi, many phospholipids, such as PA, PC, PG and PI, contain more long chain fatty acids. Furthermore, knockdown of CG4586 and CG9527 in the fat body also causes similar changes. These results coincide with the function of ACOX, which is implicated in the peroxisomal fatty acid β-oxidation pathway for catabolizing VLCFAs and some LCFAs. Similar to our findings, a previous study found that defective peroxisomal fatty acid β-oxidation resulted in enlarged LDs in C. elegans and blocked catabolism of LCFAs, such as vaccenic acid, which probably contributed to LD expansion in mutant worms [43]. Since overexpressing CG4586 or CG9527 only marginally rescues the enlarged LD phenotype of trf2 mutants, it remains to be determined whether the increased level of long chain fatty acid-containing phospholipids contributes to LD size. Regarding the regulation of fatty acid chain length in phospholipids, a recent study reported that there was increased acyl chain length in phospholipids of lung squamous cell carcinoma accompanied by significant changes in the expression of fatty acid elongases (ELOVLs) compared to matched normal tissues. A functional screen followed by phospholipidomic analysis revealed that ELOVL6 is mainly responsible for phospholipid acyl chain elongation in cancer cells [54]. Our findings provide new clues about the regulation of fatty acid chain length in phospholipids. ELOVL and the peroxisomal fatty acid β-oxidation pathway may represent two opposing regulators in determining fatty acid chain length in vivo.

Previous studies have shown that TRF2 is involved in specific biological processes including embryonic development, metamorphosis, germ cell differentiation and spermiogenesis [31, 32, 55, 56]. Our results reveal a novel function of TRF2 in the regulation of specialized transcriptional programs involved in LD size control and phospholipid fatty acid composition. Since TRF2 is conserved among metazoans, its role in the regulation of lipid metabolism may be of considerable relevance to various organisms including mammals. Our findings may provide new insights into both the regulation of lipid metabolism and the physiological functions of TRF2.

Materials and methods

Drosophila stocks and husbandry

All flies were propagated on standard cornmeal food. w1118 was used as the wild-type control. Unless specified, Drosophila stocks were obtained from the Vienna Drosophila Resource Center, the Bloomington Drosophila Stock Center, the National Institute of Genetics Stock Center, the KYOTO Stock Center and the Tsinghua University RNAi Stock Center.

Molecular biology

For gene expression constructs, the coding region of taf9, trf2 and trf2s were inserted into the transformation vector pUAST-attB through the EcoRI and XhoI sites (taf9) or NotI and XhoI sites (trf2l and trf2s). For the FLAG-TRF2s expression construct, the trf2s coding region was inserted in frame into the pUAST-attB-FLAG vector through the NotI and XhoI sites to generate pUAST-attB-FLAG-trf2s. For target gene expression constructs, the coding regions of CG9507, CG15632, CG2617, CG9486, CG10315, CG4586 and CG9527 were inserted into the pUAST-attB-FLAG vector through the NotI and XhoI sites (CG9507, CG10315, CG4586 and CG9527) or NotI and BglII sites (CG15632, CG2617 and CG9486). The coding region of all genes was amplified from w1118-derived cDNA.
Quantitative RT-PCR

Total RNA was isolated from wandering 3\textsuperscript{rd} instar larval fat body using Trizol reagent (Invitrogen) and cDNA was generated using a Superscript II reverse transcriptase kit (Invitrogen). qRT-PCR experiments were performed with a Stratagene Mx3000P system (Agilent) using Transstart Green qPCR superMix (Transgen). Relative levels of expression were normalized to \textit{rp49} in the same sample.

Generation of \textit{taf9} mutant alleles

We used imprecise P-element excision to generate \textit{taf9} mutant alleles. The starting P-element \textit{P\{GT1\}\text(y)\textsuperscript{BG00948}} harbors the \textit{white} (\textit{w}) marker gene. The \textit{TM3\Delta2–3} males which provide the transposase were mated to \textit{e(y)\textsuperscript{BG00948}} virgin females. The F1 males (\textit{e(y)\textsuperscript{BG00948}, TM3\Delta2–3}) were then crossed with \textit{FM7i} virgin females. The F2 progeny females were screened for site-directed P-element excision by the loss of the eye color marker \textit{w} and individually balanced to establish stocks. Two \textit{taf9} deletion mutants were identified by PCR from 350 balanced single-cross stocks.

Staining and microscopy

For LD staining, wandering 3\textsuperscript{rd} instar larvae were dissected in PBS and the fat body was fixed in 4\% paraformaldehyde for 30 min at room temperature. Tissues were then rinsed twice with PBS, incubated for 30 min in either a 1:500 dilution with PBS of 1 mg/ml BODIPY 493/503 (Invitrogen) or a 1:100 dilution with PBS of LipidTOX Deep Red (Invitrogen) and then rinsed twice with PBS. 2 ng/\textmu l DAPI was used to stain nuclei. Stained samples were mounted in 75\% glycerol for microscopy analysis. All images were taken using a confocal microscope. To quantify LD size, the diameters of 160 LDs (larger than 5 \textmu m that can be accurately measured) from 20 fat cells, or the three largest LDs in each of 16 fat cells, were measured by NIS-Elements BR 3.0 software.

RNA-seq analysis

Total RNA was isolated from wandering 3\textsuperscript{rd} instar larval fat body using an RNeasy lipid tissue kit (Qiagen) following the manufacturer’s instruction and 10 \mu g of total RNA was used to prepare Poly-A RNA-seq libraries. Samples were sequenced using an Illumina HiSeq 2000 sequencer at BGI TechSolutions Co., Ltd. (BGI-Tech). Sequenced reads were aligned to reference sequences using SOAPaligner/SOAP2 [57]. Genes expressed with >twofold difference and FDR (False Discovery Rate) \leq 0.001 were considered as differentially expressed for scatter plot representations and for GO term analysis using GOseq [58]. The raw sequencing data have been submitted to the Genome Sequence Archive (GSA) database with the accession number PRJCA000264.

ChIP assay and quantitative PCR

Chromatin preparation and immunoprecipitation was performed as previously described with some modifications [59]. Wandering 3\textsuperscript{rd} instar larvae were dissected and fat bodies expressing FLAG-TRF2s were collected and treated with 1\% formaldehyde in PBS for 15 min at room temperature for crosslinking. The reaction was then quenched with 125 mM glycine for 5 min and the treated samples were washed twice with PBS containing 1 mM PMSF and Protease Inhibitor Cocktail (Roche). The samples were homogenized with a cordless motor in FA buffer and the chromatin was sheared to a size range of 100–1000 bp by sonication. Cellular debris and floating lipids were removed by several centrifugation steps at 13,000 rpm for 15 min at
4°C and the supernatant was used for chromatin immunoprecipitation (ChIP). Anti-FLAG antibody (Sigma) and protein A- and protein G-agarose beads (Millipore) were used in the immunoprecipitation experiments. Anti-mouse IgG antibody (Promega) was used as the negative control. Eluted DNA was purified using a PCR purification kit (Qiagen) and then quantified by quantitative PCR. Sequences of primers used for qPCR can be found in S5 Table.

Lipidomic analysis

Lipids were extracted from wandering 3rd instar larval fat body as previously described [60]. The lipidomic analyses were carried out on an analytical system comprising an Agilent HPLC 1260 coupled with a SCIEX 5500 QTRAP. Separation of individual classes of polar lipids by normal phase HPLC was carried out using a Phenomenex Luna 3u silica column (i.d. 150x2.0 mm). Multiple reaction monitoring (MRM) transitions were set up for quantitative analysis of various polar lipids. Individual lipid species were quantified by referencing to spiked internal standards. PC-14:0/14:0, LPC-C20, PE-14:0/14:0, PS-14:0/14:0, PA-17:0/17:0, PG-14:0/14:0 were obtained from Avanti Polar Lipids and dioctanoyl phosphatidylinositol (PI, 16:0-PI) was obtained from Echelon Biosciences, Inc. Separation of glycerol lipids (DAG and TAG) by reverse phase HPLC/ESI/MS/MS was carried out on a Phenomenex Kinetex 2.6μ-C18 column (i.d. 4.6x100mm). Using neutral loss-based MS/MS techniques, the levels of TAG were calculated as relative contents to the spiked d5-TAG 48:0 internal standard (CDN Isotopes), while DAG species were quantified using 4ME 16:0 Diether DG as an internal standard (Avanti Polar Lipids). Free cholesterols and ergosterols were analyzed using HPLC/APCI/MS/MS with the corresponding d6-Cho (CDN Isotopes) as the internal standard.

Motif analysis

Promoters of target genes were analyzed using the motif discovery software HOMER [61]. We defined two types of promoter in the analysis. The core promoter is the DNA sequence from -100 to +50 (relative to +1 of the TSS) while the extended promoter is from -500 to +50. When using HOMER, we set the corresponding regions of all genes in the Drosophila genome as the background sequences. The significantly enriched motifs were selected by the criterion of q-value < 0.05 and fold change > 1.5. The q-value is provided by the HOMER output and the fold change is defined as the ratio between the number of target sequences that contain the motif and the number of background sequences that contain the motif.

Supporting information

S1 Table. RNAi phenotype of Drosophila taf genes. A summary of the RNAi phenotype and fly strains of taf genes is shown. (DOCX)

S2 Table. RNAi strains of CG4586 and CG9527 used in the study. (DOCX)

S3 Table. 141 genes tested in the RNAi screen of trf2 and taf9 target genes. (DOCX)

S4 Table. trf2 and taf9 target genes with LD phenotype. The molecular functions of trf2 and taf9 target genes with LD phenotype are listed in the table. (DOCX)

S5 Table. Primers for target gene core promoters used in ChIP-qPCR assays. (DOCX)
S1 Fig. The LD phenotype of trf2 and taf9 mutants at 2nd and early 3rd instar larval stages. BODIPY staining of LDs in the fat body of 2nd instar (A) and early 3rd instar (B) larvae from different backgrounds. There is no obvious difference between taf917 mutants and w1118 control, while trf2 mutants have large LDs. Scale bar represents 20 μm (A) and 50 μm (B), respectively.

(TIF)

S2 Fig. TRF2 regulates LD size in adult fat body. BODIPY staining of LDs in the fat body of 7-days old female adults. Knockdown of trf2 leads to more small LD. Scale bar represents 20 μm.

(TIF)

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